"Molecular investigation of pseudomonads causative of *Agaricus bisporus* blotch disease in New Zealand mushroom farms"

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ABSTRACT

Presented within this thesis is the molecular investigation of selected pseudomonad species inducing blotch disease of *Agaricus bisporus* in New Zealand mushroom farms. This work represents part of the ongoing research that is aimed at gaining a comprehensive understanding of bacterial pathogenicity toward fungi. Essentially, this thesis can be loosely defined as having two major sections, (i) population characterisation of pseudomonads able to induce blotch disease in New Zealand and (ii) the molecular investigations undertaken to understand bacterial/fungal interactions, with particular focus on elucidating induction of blotch disease of *A. bisporus*.

This thesis originally set out with the objective of investigating '*Pseudomonas gingeri*'. Prior to commencing this study, *P. gingeri* had been described as the causative organism of 'ginger blotch' disease of the commercially produced mushroom, *A. bisporus*. However, continuing observations of varying disease symptoms and varying phenotypes of bacteria isolated from 'ginger blotch' disease lesions suggested that more than one organism was causing 'ginger blotch'. A New Zealand national survey of mushrooms exhibiting disease symptoms of 'ginger blotch' was undertaken and from this, 33 pseudomonads capable of causing blotch were characterised to determine the degree of species diversity. Results identified three major findings: 1) the diversity of pseudomonads capable of causing blotch discolourations of *A. bisporus* is considerably more extensive than previously thought. 2) The organism previously described as *P. gingeri* is not solely responsible for ginger discolourations of *A. bisporus* ('ginger blotch'); and 3) a particular blotch discolouration may be caused by more than a single pseudomonad species. The result of these findings affecting the classification of previously identified *P. gingeri* is discussed. Furthermore, the isolation and characterisation of a novel *Pseudomonas* isolate, NZI7, is reported that exhibited all phenotypic criteria described for the identification of *P. tolaasii*, yet showed no genetic similarity. The impact of NZI7 identification is that it demonstrates limitations to the previously accepted phenotypic techniques for *P. tolaasii* identification.

The observation of many different blotch discolourations being caused by several different *Pseudomonas* species, termed blotch causing organisms (BCOs), altered the subsequent research direction to a holistic approach to determine genetic determinants involved in colonisation and blotch formation amongst these diverse BCOs. Phenotypic analyses of putative pathogenicity determinants (PPDs) described in other virulent bacteria were performed on all 33 BCOs including; auxotrophy, biofilm formation, protease, lipase and chitinase production. Abiotic biofilm formation under varying conditions was the only consistent PPD phenotype shown by all BCOs.

As it was not feasible to carry out detailed investigations on all 33 BCOs, a single BCO *P. putida* NZ103 was selected for Tn*5* mutagenesis to identify the role of NZ103 PPDs in blotch discolouration.

From a total of 5000 mini-Tn*5*km*lacZ*2 transposon generated NZ103 mutants, 45 were selected based on deficiencies in one or a combination of the PPDs analysed. Furthermore, these 45 NZ103 mutants were assessed using *in vitro A. bisporus* tissue bioassays and from these, 13/45 had substantial blotch reduction. Ten isolates were chosen from these NZ103 blotch reduced mutants and the genetic regions flanking the mini-Tn*5*km*lacZ*2 insertion point were characterized by restriction mapping, subcloning, nucleotide sequencing and comparison to entries in the BLAST database. Genetic similarities of genes interrupted in these 10 NZ103 mutants included: genes encoding two-component regulatory systems such as *gacS/lemA/rtpA*; proteins involved in transcriptional regulation (such as the LysR family); probable 2-hydroxyacid dehydrogenase involved in energy metabolism; a possible integron gene; outer membrane protein OprF involved in osmoregulation and cell shape; a metalloprotease secretion gene *aprE*; and several extracellular binding receptors which may serve as chemoreceptors. These putative identifications and their relationship to current literature is discussed with a focus on interpreting how such genes may be involved in either bacterial/fungal interactions and/or blotch formation of *A. bisporus*.

A summary of all results and discussions within the scope of this thesis is presented within a number of 'models' that have been created to encompass critical factors involved in blotch disease and pose questions for future research to build upon. Finally, Chapter 8 emphasises areas for future research that would further the understanding of bacterial/fungal interactions as well as benefit management of mushroom blotch diseases.

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LIST OF ABBREVIATIONS

bp, kb	base pair, kilo base
dsDNA	double stranded DNA
rRNA	ribosomal RNA
hr, min, sec	hour, minute, second
rpm	revolutions per second
ddH_2O	double distilled water
g, kg	gram, kilo gram
AA(s)	amino acid(s)
Bfm^+, Bfm^-, Bfm^R	biofilm positive, biofilm minus, biofilm reduced
ml, L	milli litre, litre
$cfu.ml^{-1}$	colony forming units per ml
MHz	mega-hertz
nm	nanometer
ppm	parts per million
UV	ultraviolet light
M	Molar concentration
pM	pico molar
v/v	volume per volume
w/v	weight per volume
LB, LBA	Luria bertani media, LB agar
KB	Kings media 'B'
PVC	Polyvinyl chloride
LPS	Lipopolysaccharide
EPS	Exopolysaccharide
BCO(s)	Blotch causing organism(s)
BIF(s)	blotch inducing factor(s)
°C	degrees Celsius
OD_{xxx}	optical density at wavelength of xxx nm
ca.	approximately
ORF	open reading frame
LDP	lipodepsipeptide
PPD(s)	putative pathogenicity determinant(s)

FOREWORD ON THE STRUCTURE OF THIS THESIS

Overall structure of thesis

This thesis is structured so that the first chapter gives an overall introduction and literature review of *Agaricus bisporus* physiology and aspects of commercial growing deemed important to the development of blotch diseases. Subsequent chapters contain focused introductions to the specific research carried out within that chapter and then provide experimental results with a discussion of the research findings obtained. A concluding chapter is provided at the end of the thesis in which all research carried out is summarised with a holistic analysis that encompass and discuss the significant contextual placement of this research within current knowledge.

Experimental procedures

I have chosen to describe all commonly performed experimental procedures used in this study at the end of the thesis, within Appendix I so as to avoid repetition and reporting well-described methodology. However, any methodology devised within this study has been included within the respective chapters.

Mushroom farm confidentiality

Mushroom farms within this thesis are commercial farms in competition with each other in New Zealand. Kind provision of samples took place due to their generosity, however, the Commercial Mushroom Growers Federation (NZ) Ltd requested that company names not to be associated with isolation of disease statistics. Therefore, within this thesis farms are either referred to as 'minor' or 'major' farms out of courtesy.

PUBLICATIONS DURING THIS THESIS

Related to the Pseudomonads studied in this thesis

Godfrey, S.A.C., Harrow, S.A., Marshall, J.M., and Klena, J.D. (2001). "Characterisation of pseudomonads causing ginger blotch disease of cultivated *Agaricus bisporus* using 16S rRNA sequence analysis". *Applied and Environmental Microbiology*, **67**: 4316-4323.

Godfrey, S.A.C., Marshall, J.M., and Klena, J.D. (2001). "Genetic characterisation of *Pseudomonas* "NZI7" - a novel pathogen that results in a brown blotch disease of *Agaricus bisporus*". *Journal of Applied Microbiology*, **91**: 412-420.

Publications as a result of the methodology gained in this thesis

Godfrey, S.A.C., and Marshall, J.M. (2002). "Identification of *Pseudomonas viridiflava* and *P. marginalis* isolates causative of carrot post harvest bacterial soft rot during refrigerated export from New Zealand". Plant Pathology, 51 155-162.

Godfrey, S.A.C., and Marshall, J.M. (2002). "Characterisation of pseudomonads entering New Zealand in exotic soil attached to shipping containers.". New Zealand Journal of Crop and Horticultural Science, 30 19-27.

Manuscripts in preparation from the results in this thesis

Godfrey, S.A.C. (2003). "Pathogenicity determinants of pseudomonads causative of mushroom diseases - a review". In preparation.

Godfrey, S.A.C., Monds, R.D., Marshall, J.M. and Klena, J.D. (2003). "Transposon identified pathogenicity determinants of pseudomonads causing blotch disease of *Agaricus bisporus*". In preparation.

Godfrey, S.A.C., Monds, R.D., Marshall, J.M. and Klena, J.D. (2003). "OprF involvement in *Pseudomonas putida* NZ103 colonisation of *A. bisporus*". In preparation.

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Mushroom growers of the common button mushroom, *Agaricus bisporus*, are constantly battling abiotic and biotic factors that prevent maximum cropping yields of marketable quality mushrooms. In modern cropping systems, abiotic factors such as temperature, humidity, substrate composition and water content are largely controllable. However, biotic factors tend to be more dynamic, less manageable and their presence often cause significant crop reductions. Unfortunately the mesophilic, humid abiotic conditions used for optimal mushroom growing are also favourable for other mesophilic organisms, including insect, viral, fungal and bacterial pathogens. Non-aseptic composting and mushroom cultivating conditions using primary raw materials from other industries provide numerous opportunities for contamination and re-infection by deleterious organisms. Of the biotic factors regarded as detrimental to mushroom crops it is the bacterial genus causative of blotch disease, the pseudomonads, that are the focus of this thesis. Spurred on by continual problem of quality loss, bacterial blotch of *A. bisporus* continues to be studied internationally in an attempt to elucidate the mechanisms of disease with the ultimate goal of efficient blotch management.

1.1 *Agaricus bisporus*

World-wide production of cultivated mushrooms continues to grow each year. In 1997 the annual world production of cultivated mushrooms was 6.34 million metric tones, compared with only 4.92 million metric tones in 1994 (Courvoisier, 1999). The production of the button mushroom, *Agaricus bisporus* (Lange) Imbach, or "champignon' in Europe is approximately 8-9 x 10^5 tonnes per year. France and The Netherlands are the countries with the highest production of mushrooms (2-2.3 x 10^5 tonnes), followed by UK, where the mushroom crop is the most lucrative protected horticultural commodity with an annual value in excess of 300 million Euro. New Zealand has a production of 7.3 x 10^3 tonnes a year at an estimated value of five million NZD.

The white colour in mushrooms is considered as the most important quality parameter. White and off-white mushrooms still have by far the largest market share in the Western hemisphere (80%).

Discolouration of white mushrooms make them less attractive to the consumer and therefore cause yield losses of marketable cro*P*. Discolouration in the fruitbodies is usually perceived as a senescence symptom or as a symptom of microbiological deterioration (spoilage). Bacterial blotch diseases produced by pseudomonads have been described worldwide for many years and cause severe tissue discolourations.

1.1.1 Taxonomy of *A. bisporus*

The common button mushroom or 'Champignon', was classified for the first time by Lange (1926) (cited by (Foret, 1989)) as *Psalliota hortensis* var. *bispora*. Later Imbach (1946) (cited by (Foret, 1989)) transferred the genus *Psalliota* to *Agaricus*. With the start of cultivation, many other names were given such as *Psalliota arvensis*, *Psalliota hortensis*, *Psalliota bispora*, *Agaricus campestris* (Singer 1961 cited by (Foret, 1989)), *Agaricus arvensis* and *Agaricus hortensis*. Taxonomically the name *Agaricus brunnescens* is more adequate (Stamets and Chilton, 1983) but the most common name currently is *Agaricus bisporus*.

Agaricus bisporus belongs to the kingdom fungae, division of Eumycota, subdivision of basidiomycotina, class of basidiomycetes, subclass of Autobasidiomycetes, order of Hymenobasidiomycetes, family of Agaricaceae, and the genus of *Agaricus* (Carlile and Watkinson, 1994). In natural conditions, the brown variety of *Agaricus* is var. *bisporus*, the white is var. *albidus* and the cream variety var. *avellaneous* (Stamets and Chilton, 1983). Mycelium represent the vegetative state and sporophores that have macroscopic dimensions is the reproductive part able to produce spores.

The name of *Agaricus* stems from the Greek word 'agarikon' which scholars believed to originate from a Scythian tribe called Agari who were well versed in the use of medicinal plants and employed a fungus called 'agaricum', probably a polypore in the genus *Fomes*. The species spelling brunnescens comes from the Latin 'brunneus' or brown. Literally, the name means the fungus that becomes brown, probably referring to the colour change of the flesh upon bruising. The name *bisporus* indicates the two-spored basidia populating the gill surface.

Historically, this species and/or its close relatives were the first mushrooms to be cultivated in Europe during the late 1700's. Today, a broad range of commercially available strains exists, many of which have been genetically selected for certain advantageous characteristics, especially yield, colour and structure (Stamets and Chilton, 1983). For commercial reasons spurred by consumer demand, growers produced white hybrids of *A. bisporus*. *A. bisporus* remains the most widely cultivated mushroom in the world today and it is the white button mushroom that remains the most predominant mushroom sold in the western world. However, the brown button mushrooms are having a resurgence in popularity.

A. bisporus is naturally found in soils enriched with dung, on compost piles and in horse stables. It is a widely distributed temperate species that fruits during environmental conditions provided by

autumn. These specific environmental conditions that promote the formation of the sexual mushroom state, occurs naturally once a year. It is these conditions that mushroom growers endeavor to artificially replicate within growing sheds to establish continual mushroom cropping all year round.

1.1.2 Morphological structures

The biological life of mushrooms follows a cycle composed of three different structures: spores, mycelium (vegetative structure) and sporophore (sexual reproductive structure). Spore germination produces a mycelium, the mycelia produce a sporophore (or fruitbody) and the sporophore produces spores, thus closing the cycle.

Mycelium

Upon germination, the fungal spore germinates to produce a long thread, or hypha, that consists of multinucleate cells containing cytoplasm contained within a rigid cell wall composed of chitin and cellulose. Elongating hyphae will branch repeatedly to form an integrated mass that constitutes the mycelia. Fungal growth is characteristically confined to the tips of the hyphae and growth of hyphae is usually uniform in diameter. Mycelial growth is proportional to nutrient availability and will occur until conditions are favourable for the macroscopic formation of sporophores.

Sporophores (or fruitbodies)

The mycelia can be stimulated to produce sporophores by changes in environmental conditions. One of the most dramatic growth phenomena observed in nature is the rapid emergence and expansion of the mushroom sporophore. The sporophore is a macroscopic structure that exists to enable sexual reproduction to occur (Figure 1-1).

Sporophores are composed of closely packed hyphae with the stalk of *A. bisporus* species appearing short and thick (compared with other mushrooms). The cap is characteristically brown, white or cream. The under-side of the cap consists of rows of radiating gills that are pink when young, but darkening to sepia and then chocolate brown with age. A skin or epidermis covers and protects the gill in early mushroom development, but when the mushroom matures, the epidermis breaks to expose the gills. The broken skin at the lower part of the cap is called velum, and it remains as a persistent membranous annulus on the stalk (Stamets and Chilton, 1983).

The gill is formed by thousands of basidia that are specialised enlarged hyphae that function for spore (basidiospore) formation and is where meiosis occurs. Then, a slender projection known as a sterigma develops at its upper end, and two different nuclei (of the four formed) migrate into the sterigma as the latter enlarges. Eventually, a cross wall is formed near the base of the sterigma and the cell thus is cut off being termed a basidiospore. The same process is repeated for the remaining other two nuclei in the basidium, so that a mature basidium bears on its surface two basidiospores (thus the so-named *bisporus*). The basidia project horizontally from the vertical walls of the gill, and

consequently when the basidiospores are ejected, they pass into the air space between adjacent gills and fall to the ground below. The spores of the commercial *A. bisporus* strains are chocolate brown.

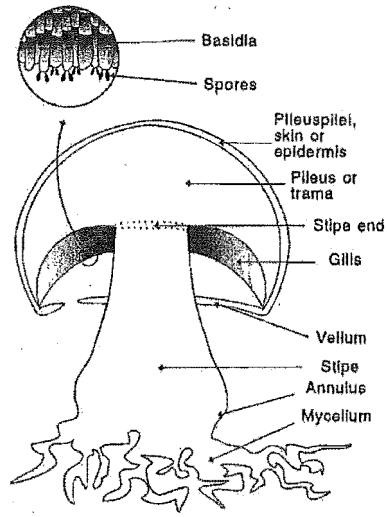

Figure 1-1 Schematic representation of sections of the *A. bisporus* sporophore.

A. bisporus mycelia grow beneath the soil and it is not until the reproductive sporophore or fruitbody (Figure 1-1) emerges from the soil that the 'mushroom' is visible above the surface. Although fruitbody development from primordia to mature sporophore is a continual process (Hammond and Nichols, 1975), it can be defined into seven stages for experimental purposes (Figure 1-2). The button mushroom is usually harvested for the market at developmental stage 2 and the flat mushroom at stage 6.

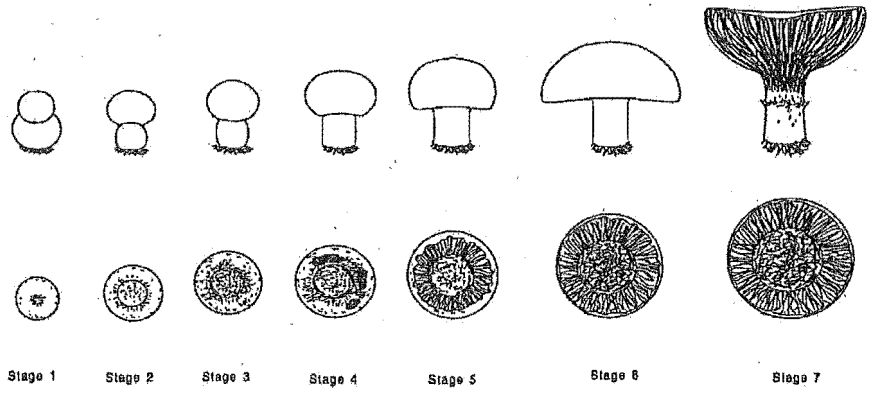

Figure 1-2 Development stages of *A. bisporus* (Hammond and Nichols, 1975). (Stage 1) "pinhead", it is characterized by undifferentiated velum (diameter of the cap approx. < 5 mm); (Stage 2) "button", it is characterized by visible and intact but not stretched velum (diameter of the cap approx. 20-30 mm); (Stage 3) "closed cup", the velum is stretched but still intact (diameter approx. 30-40 mm); (Stage 4) "cup", velum starting to tear (diameter approx. 30-40 mm); (Stage 5) "cup", velum torn, pileus still cup shaped, gills clearly visible (diameter approx, 30-50 mm); (Stage 6) "flat", it has the upper surface flat of pileus convex, gill surface fiat or slightly concave (diameter approx. 40-60 mm); and (Stage 7) 'flat", the gill surface curving upwards (diameter approx. 50-70 mm).

The two life cycles

A. bisporus is capable of developing two different life cycles. One life cycle is called homothallic and the other heterothallic (amphithallic life cycle). *A. bisporus* normally produce bisporic basidia (81%), which form two basidiospores with two different nuclei inside each spore (heterokaryotic spores) (Stanier, *et al.*, 1979). When spores germinate, they develop heterokaryotic mycelium (two nuclei in the cytoplasm) which is sufficient to produce fertile sporophores that confer a predominant secondarily homothallic life cycle (Callac, *et al.*, 1996) (Figure 1-3).

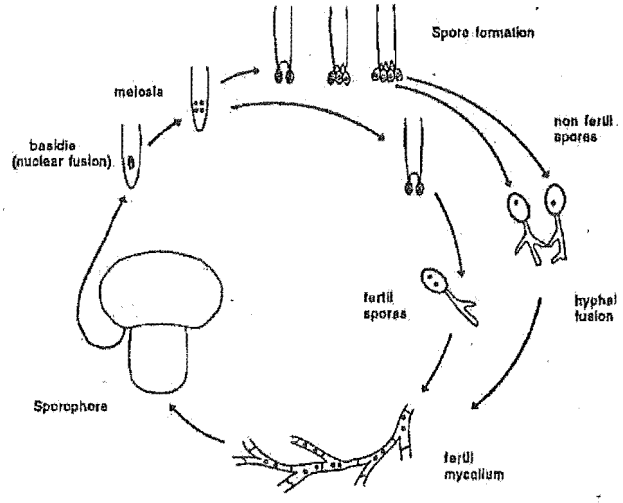

Figure 1-3 The double life cycle of *A. bisporus* (Foret, 1989)

At a lower percentage, the basidia can also be trisporic (18%) or tetrasporic (1%). The tetrasporic basidia produce four spores with only one nucleus (haploid) that can give rise, on germination, to homokaryotic mycelium (only one nucleus in the cytoplasm) that are incapable of fructifying. However, if two such homokaryotic mycelium of compatible mating types come into contact, hyphal fusion followed by nuclear exchange takes place, to produce a still haploid heterokaryon; the two kinds of nuclei become associated in a very regular fashion, one pair occurring in each compartment of the septate mycelium. During growth of the heterokaryon, the two kinds of nuclei divide synchronously. A heterokaryotic mycelium may continue to grow vegetatively for a long time, and during such vegetative growth, fusion between the paired nuclei never occurs. Fusion takes place only at the time of fructification, when the basidia are produced and is followed in each basidium by immediate meiosis to produce the haploid or diploid nuclei destined to enter the basidiospores. This is classified as the heterothallic life cycle of *A. bisporus* (Figure 1-3).

The trisporic basidia produce two haploid and one diploid basidiospore that can give rise to the two different mycelium and to the homothallic and heterothallic life cycles (Carlile and Watkinson, 1994).

1.1.3 Cultivation

Mushrooms have been cultivated by mankind for many years and the procedure is well standardized. In 1630, in France, it was found that mushrooms could develop on suitably prepared beds of horse manure in gardens. A few years later, mushrooms were cultivated in caves where the constant environmental conditions increased crop yields and cultivation was no longer dependent on seasons and weather. Today, mushroom cultivation is carried out in specialised mushroom houses, with artificial control of optimal environmental parameters such as relative humidity (RH), CO_2, temperature and aeration.

Generalised overview of mushroom cultivation

Many different mushroom growers in many different countries have subtle variations on growing mushrooms. In reality, mushroom cultivation requires an extensive knowledge on the means of controlling biotic and abiotic factors. Adaptation to disturbances in these factors ultimately determines the success or otherwise of mushroom cro*P*. However, for the purpose of this review, generalised procedures used in mushroom cultivation are described (Van Griensven, 1988).

Cultivation conditions

Two phases are essential in mushroom production, each of which is a solid substrate fermentation. The first is composting, the preparation of a suitable growth medium, and the second is the growth of *A. bisporus* until harvesting of mushrooms (Flegg, *et al.*, 1985). A mushroom farm implements a rotational production plan so that a crop of mushrooms is started every week to ensure continual picking. A summary of the processes of commercial mushroom production is depicted in Figure 1-4, and following this is a more detailed account of each process.

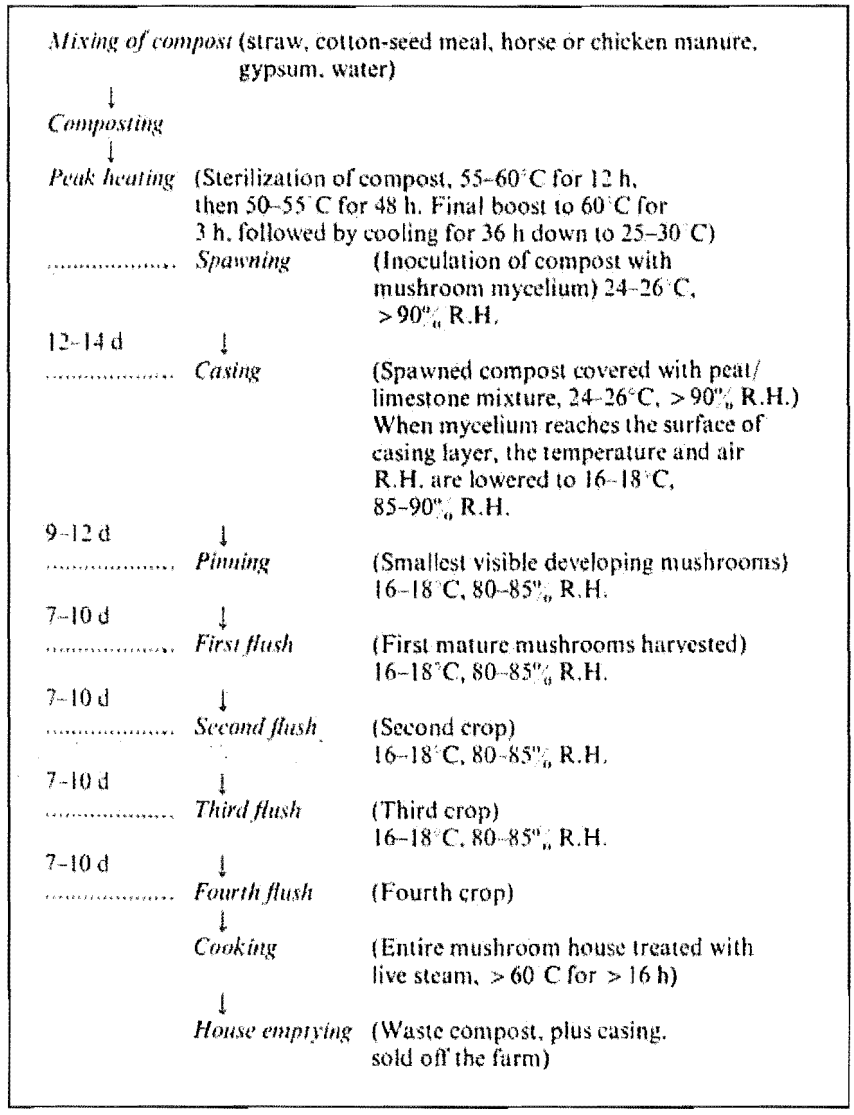

Figure 1-4 Generalised flow diagram of the processes of commercial mushroom production (Wong and Preece, 1980).

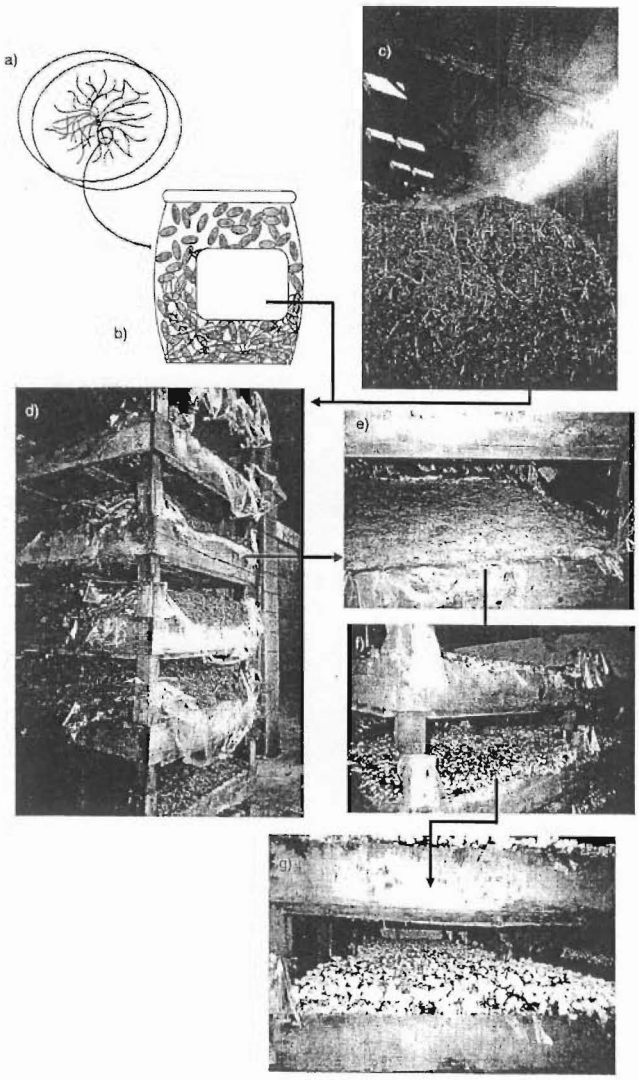

Figure 1-5 Cultivation outline of commercial *A. bisporus*. The suitable mushroom strain (A) is grown in sterile conditions on grain (B). Growers prepare the compost (C) and inoculate it with spawn (D). When the compost is fully-grown with mycelium (E) a casing layer is added to initiate the fruitbody formation (F) that results in a flush of mushrooms (G).

Composting

The materials used for preparing mushroom compost vary, but wheat straw and manure (either chicken or horse) are generally utilised (Flegg, *et al.*, 1985). Since several tons of material will be processed daily, bulk-handling machinery is needed and sterile conditions are difficult to maintain. The materials are mixed, made into stacks, and watered to provide damp but not waterlogged conditions. A dense flora of bacteria ($1x10^{10}$ cfu.g^{-1}) and fungi ($1x10^{6}$ cfu.g^{-1}) develop and temperatures within compost stacks may reach 70°C, but excessive heating and anaerobic conditions are avoided by frequent turning. Thermotolerant microorganisms, such as thermophilic actinomycetes of the genus *Streptomyces* and the thermotolerant fungi of the genus *Humicola*, decompose cellulose

and produce vitamins influencing advantageously the growth of mushroom mycelia (Hayes, *et al.*, 1969). Therefore, the aim of composting is to eliminate low molecular weight nutrients and produce high molecular weight materials such as cellulose and lignin. Low molecular weight nutrients can be utilized by many undesired microorganisms, whereas high molecular materials are specifically utilized by *A. bisporus*, thus providing an environment selective for mushroom culturing.

'Peak heat'

Composting is completed by a process referred to as 'peak heating' that is essentially pasteurization in special chambers at 60°C. Peak heat drives off ammonia (which is toxic for *A. bisporus* growth) and has a desired two-fold biotic effect: selection for the growth of thermophilic microorganisms; and elimination of growth of mesophilic organisms.

The benefit of thermophilic organisms

The role of thermophilic fungi in the production of mushroom compost for the growth of *A. bisporus* is without doubt invaluable and is of fundamental importance in determining compost selectivity (Ross and Harris, 1983, Straatsma, *et al.*, 1989, Op Den Camp, *et al.*, 1990, Wiegant, *et al.*, 1992). Thermophilic fungi continue to provide complex polysaccharides used by *A. bisporus*.

Elimination of mesophilic organisms

The temperatures reached at peak heat are sufficient to eliminate the mesophilic microflora that might later infect and/or compete with establishing *A. bisporus* mycelia. Because of the bulk handling of compost, it is realistic that not all compost will be subjected to the same temperature profiles and thus incorrect peak heat management is one of the major sources of introduction of contaminating microorganisms that cause crop losses.

Spawn

Strains of *A. bisporus* with commercially attractive characteristics such as aesthetic qualities and ease of cultivation are maintained by specialist companies. Monocultures of *A. bisporus* mycelia are laboratory cultivated on cereal grain and supplied to growers and are termed mushroom 'spawn'. The use of aseptically prepared mono-cultures of spawn for inoculation minimises the transmission of pathogens of the mushroom, such as bacteria causing blotch (*Pseudomonas tolaasii*, *P. gingeri*), fungi (*Verticillium, Trichoderma*) and virus (la France, Virus X). Subsequent handling, however, must still be hygienic, to avoid later infections. Sometimes, beds must be watered with fungicides or chemicals to avoid the further development of diseases.

Spawning compost

'Spawning' is a term that refers to the inoculation and mycelial colonisation of compost with the desired mushroom spawn. Spawn is mixed into the compost using specialised machinery, put into trays and then maintained at 25°C (the optimal temperature for mycelial growth) (Figure 1-5).

Casing

The composition of the casing is different from one farm to another, but mostly it is composed of peat, limestone and chalk. Casing is designed primarily to have high water retention properties and provide a substrate that facilitates pinning of the mushroom mycelia. When the compost is fully colonized (1.5 to 2 weeks) it is covered with a 3 to 10 cm layer of a casing soil and the trays are then placed in a cropping room at 18°C, a temperature that promotes fruitbody initiation and development (Figure 1-5) It is thought that microorganisms from the casing layer are necessary for the initiation of the primordia of fruit bodies[1].

Flushes

Once the casing is colonized by the mycelia and environmental conditions have been deliberately altered to induce pinning, pinheads will grow into fruitbodies (or sporophores) after approximately 3 weeks (Figure 1-5). This concerted growth of fruit bodies is described as a flush. Once the first flush has been picked, environmental conditions are again altered to induce further pinhead development for subsequent flushes. Usually further flushes of fruit bodies occur at 7-10 days intervals for about a further 4 weeks. It is typical for farms in New Zealand not to exceed three flushes, although some farms carry out up to five flushes and overseas, up to 8 flushes. However, it is generally considered to be not cost effective to carry out too many flushes because the yield and quality of the mushrooms decrease due to depletion of nutrients and establishment of diseases. Disease establishment occurs over time and as well as mushroom yield reduction in specific crops, establishment leads to potential long-term farm contamination.

Cook out

Following the completion of desired flushes, the cropping room is then treated with steam at 70°C for 12 hours, the rooms cleared and cleaned. This process, termed 'cook out' is designed to kill pests and pathogens that may have established during the cropping cycles. Cook outs are implemented as a farm management process designed to prevent disease spreading from on cropping room to another and/or around the farm. Often spent compost is sold as compost fertiliser for gardens.

Summary of mushroom cultivation

Essentially what has been described above is a simplistic overview of mushroom cultivation. One important observation is the underpinning process revolves around selecting for the growth of desired microorganisms, while at the same time selecting against the growth of unwanted pests and pathogens. Mushroom cultivation, and in particular the composting process, is a dynamic microbiological ecosystem in which much can go wrong (Ishii, *et al.*, 2000).

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[1] Beneficial microorganisms are discussed in Sections 1.3.2

1.1.4 Chemical composition of *A. bisporus*

Mushrooms are a good source of nutrients but assessment of chemical composition has been impeded by the fact that values vary not only from study to study, but also between flushes within a given crop. It is suspected because mushroom development is dependant on the nutrients in their growing environment. Therefore, variability and availability of nutrients and environmental conditions are all important factors affecting the chemical composition of individual fresh mushrooms. However, various studies have been carried out and compositions have been determined.

The major component (89-94%) of fresh mushrooms is water (Laborde and Delpech, 1991) along with 2.5 - 5.8% of carbohydrates, 2.6 - 4% of proteins, 0.2 - 0.7 % of crude fat, 0.6 - 1.1% of fiber, and more than 1% of ash (Mau, *et al.*, 1993). *A. bisporus* white and brown mushrooms essentially have the same basic composition as each other with chitin as the source of dietary fiber and each having 27 kcal per 100g of energy (Mattila, *et al.*, 2002).

The major soluble carbohydrates are mannitol (30 - 50% of dry weight (dw)), trehalose and glycogen. Other carbohydrates found in mushrooms include sucrose, glucose, hemicellulose and chitin (5-10% of dw), which is the major structural component found in the cell wall. The carbohydrate composition varies with the flushing pattern. Mannitol and trehalose vary depending on the stage of growth of the mushroom (Beelman and Edwards, 1989).

Neutral lipids have been shown to be approximately 50% of the total lipid fraction and contained both unsaturated and saturated lipids. Mushrooms are also rich in the essential fatty acid linoleic acid (63 - 74% of total fatty acids) (Mau, *et al.*, 1993).

Protein measurements are complicated by the presence of non-protein nitrogen-containing compounds (Braaksma and Schapp, 1996). However, mushrooms contain both protein and non-protein amino acids and compared to most common vegetables, are a source of almost all essential amino acids (Mattila, *et al.*, 2002). In other studies, amino acid composition was shown to vary depending upon flush number and compost composition (Foret and Arpin, 1991) but protein content remains constant between flushes (Beelman and Edwards, 1989).

Mushrooms contain large amounts of potassium, phosphorous, copper and iron but do not contain appreciable quantities of calcium and it has a low sodium content. Mushrooms also supply significant quantities of other elements including manganese, molybdenum and especially zinc (Mau, *et al.*, 1993) and are also considered to be a good source of the vitamins riboflavin and niacin (Beelman and Edwards, 1989).

1.1.5 Quality of fresh mushrooms

Mushrooms deemed to be of a high quality are completely white, should not have elongated stipes, and gills should not be visible. It should also have a tender, but not soft texture and have the characteristic mushroom odour. Fresh mushrooms are highly perishable and have a shelf life of 2-3

days after harvest at room temperature. Quality is later greatly influenced by storage temperature, mechanical damage and to a lesser extent by flush number (Burton and Nobel, 1993).

Commercially defined mushroom quality declines rapidly on the bed due to the natural maturation process initiated for the natural development and production of spores (Burton, *et al.*, 1995). For commercial purposes, mushrooms are usually harvested at the button stage (Figure 1-2) because they are reported to have a longer shelf life than mushrooms in the open cap stage of development (Mau, *et al.*, 1993).

Prior to harvest, nutrients and water from the compost, and casing material sustain sporophore growth but quality declines after harvest due to the senescence processes. When a sporophore is harvested, the nutrient and water supply are cut off and its metabolism has to adapt to sustain gill growth and spore production (Tsai, *et al.*, 1974, Burton, 1993).

Parameters that define mushroom quality

Colour

White mushrooms without dark lesions or bruising are undoubtedly the most important aspects of defining fresh *A. bisporus* quality. However, mushrooms are by nature very sensitive to browning (Burton, 1986) which occurs due to the oxidation of phenolic compounds into brown melanins by polyphenol oxydases such as tyrosinases (Martinez and Whitaker, 1995). The browning process will be reviewed in further detail in (Chapter 1.2).

The most problematic factor that induces discolouration is microbial colonization of the mushroom cap. Microorganisms able to induce mushroom cap discolourations are well studied because discoloured spots and sunken areas are unacceptable for a high quality mushroom. *Pseudomonas tolaasii, P. gingeri, P. agarici, Verticillium* and *Trichoderma* are some well characterised microorganisms that can discolour a white mushroom.

Appearance and texture

After harvest, fruitbodies continue to develop and carry on the biological processes such as respiration, converting previously stored substances into energy, carbon dioxide and water. Much of the energy yielded by respiration is used in the ongoing processes of maturation. In general, the higher the rate of respiration, the faster the processes of maturation and senescence proceeds. Maturation leads to aesthetically undesirable traits for marketability including expansion and opening of the cap, elongation of the stipe, and shedding of the reproductive spores (Mau, *et al.*, 1993). Mannitol is the non-structural carbohydrate of the sporophore mostly used during respiration to obtain the requested energy (Hammond and Nichols, 1975). Apart from the energy extracted from mannitol, expansion of the pileus by growth of gills and postharvest elongation of the stipe is also supported by increased cell wall chitin and protein (Burton, *et al.*, 1993).

Senescence includes changes in the texture because cell degeneration is produced. At harvest, mushrooms grown properly are firm, crisp (resist deformation) and tender (easy to shear or chew), but under postharvest conditions deterioration causes both softening and toughening. This is the result of chitin synthesis in cell walls (toughening) and loss of cell turgor due to changes in membrane permeability (softening) (Burton, 1988b).

The relative humidity of the environment in which mushrooms are stored greatly affects the loss of water. Dehydration has been correlated with blackening of stipes and with opening of veils, both of which are traits undesirable aesthetic qualities.

Aroma and flavour of mushrooms

The aroma and flavour of mushrooms are important in defining quality. The flavour substances of mushrooms can be classified into: non-volatile components such as amino acids and nucleotides, and volatile compounds such as 1-octen-3-ol and 3-octanone. The basic amino acid fraction of mushrooms, consisting of alanine, arginine, aspartic acid, glutamic acid, glycine, and lysine, is probably responsible for some aspects of flavour, but all nitrogenous components contribute to some extent to the typical mushroom flavour. In addition, mushrooms contain relatively high levels of free glutamic acid, which is known to be a flavour enhancer. Non-nitrogenous compounds are also present in mushrooms and undoubtedly contribute to the overall flavour. Purine bases and 5'-nucleotides may contribute to the 'meaty' component of mushroom flavour.

The most characteristic aroma may be attributed to a series of eight-carbon (C8) volatile compounds common to most mushroom species. In *A. bisporus* the most important are 1- octanol, 3-octanol, 3-octanone, l-octen-3-ol, 2-octen-l-ol and l-octen-3-one. In addition, benzaldehyde and benzyl alcohol were also found and are possibly the cause of 'almond- like' aroma.

During postharvest storage, the 1-octen-3-ol content and the activity of lipoxygenase and hydroperoxide lyase enzymes decreased over time. Some compounds, such as free amino acids and nucleotides, are produced as a result of the increased activities of protease, ribonuclease and deoxyribonuclease. All these complicated processes may contribute to the characteristic flavour of mushrooms (Mau, *et al.*, 1993).

Methods of improving quality

Irrigation treatments with calcium chloride, oxine, or gypsum (Mau, *et al.*, 1993), modified atmosphere packing (Burton, 1991, Burton, 1993), irradiation with a low dose of γ-irradiation (Smierzchalska, *et al.*, 1988), stipe trimming (Mau, *et al.*, 1993), washing treatments with antioxidant chemicals and storage at low temperatures (Burton and Nobel, 1993) in humid rooms are methods actually used to preserve mushroom quality. At present, new methods are being tested to improve the quality such as selection of new strains, browning inhibitors, compost composition and nutrient addition during cultivation (Olivier, *et al.*, 1997, Sapers, *et al.*, 2001).

Main enzymes involved in loss of quality

In the basidiomycetes, there is evidence for the regulatory role of proteases in the senescence processes such as activation of chitin synthase from *A. bisporus* (Burton, *et al.*, 1994). Proteases are known to be involved in fungal tissue autolysis and so contribute to a loss of sporophore texture and therefore quality. Mushroom quality can also be influenced by the proteolytic activation of tyrosinase, the enzyme responsible for tissue browning (Burton and Nobel, 1993).

The changes in activity during sporophore development or maturation depended on the period in the flushing cycle when the sporophore was initiated (Burton, *et al.*, 1994). A large increase in proteolytic activity and a decrease in protein content was observed in the tissues of harvested mushrooms after five days storage at 18°C (Burton, 1988b).

1.2 Physiological discolouration in *A. bisporus*

The physiological discolouration of *A. bisporus* has been widely studied due to its impact on high value aesthetic quality of product. The biochemical components of the major discolouration processes are discussed here as these processes can be induced by microbial pathogens.

Discolouration in mushroom sporophores is caused primarily by the oxidation of phenolic substances, catalysed by polyphenol oxidases (PPO). PPOs and its substrates are prevented from reacting within the intact cell (Burton, 1986). However, when mushroom cells are damaged (such as by bruising, senescence, infection or maturation) the cellular contents mix with each other, the phenols are liberated to the cytoplasm, and oxygen penetrates in the intercellular environment initiating enzymatic oxidation. Enzymatic oxidation results in the synthesis of pigmented melanins that are a major cause of *A. bisporus* discolouration.

1.2.1 Melanins

Dark brown to black pigments occur widely in fungi, usually in the sexual spores or their enclosing structures. Fungal melanins occur either in cell walls or as extracellular polymers formed in the medium around fungal cells. Melanins have not generally been found in fungal cytoplasm, although it has been suggested that melanin precursors may be secreted from the cytoplasm to the cell wall where they are oxidized to melanin (Margalith, 1992).

It is these pigmented melanins that are produced in response to cellular damage of *A. bisporus* tissue and will result in mushroom hyphae exhibiting a wide range of discolourations from yellow, red purple to brown.

Physiological function of melanins

Essentially, the primary role of melanins in mushroom tissue is that of protection. It was suggested that hyphae or specific highly pigmented structures might exhibit resistance to a variety of adverse

environmental factors during prolonged periods of unfavorable growth conditions. When fungi are exposed to extreme temperatures it is suggested that the insoluble, hydrophobic, and rigid aromatic nature of melanins provides resistance to dehydration and heat (Arpin and Bouillant, 1988). Melanized fungi possess increased resistance to microbial attack as well as enhanced survival over its competitors. The polyaromatic material of the pigment may also act as an inhibitor of cell wall degrading enzymes. When attacked by microorganisms, the non-melanized cells and the non-melanized portions of the cell walls are usually lysed, whereas the melanized portion of the cells remains intact. It was also suggested that melanin may combine with, or overlay, the lysis susceptible surface components in such a way as to prevent enzymes from combining with their potential substrates (Margalith, 1992).

Furthermore, melanin-containing spores were found to be more resistant to killing by ultraviolet light or solar irradiation. Melanins are thought to absorb various types of radiation and dissipate energy primarily by undergoing reversible increases in free radicals. Hence, melanized cell walls protect the cell cytoplasm from the damaging effects of free radicals formed by irradiation and thus protect cells against oxidizing conditions (Arpin and Bouillant, 1988).

Melanins - structure and chemical properties

Melanins represent a class of dark pigments that result from oxidative polymerization of various types of phenols. Melanins are insoluble in water, HCl, and most organic solvents and are extremely resistant to enzymatic and chemical degradation. These combined chemical properties make structural elucidation difficult (Swan, 1974, Zeise and Chedekel, 1992). Melanins display a wide range of chemical properties by acting as effective redox polymers, ion exchangers and radical scavengers, and by showing a strong tendency to bind with aromatic and lipophylic compounds (Margalith, 1992).

Melanin pigments have been classified as eumelanins, phaeomelanins and allomelanins. Eumelanins are black insoluble pigments derived from tyrosine while phaeomelanins are not dark brown or black but have a reddish appearance and were found to contain cystein and derivatives of tyrosine. Allomelanins are described mostly in lower organisms, having no tyrosine, but contain a variety of nitrogen-free precursors, such as poly-hydroxynaphthalenes (Bell and Wheeler, 1986).

1.2.2 Tyrosinase

Tyrosinase is one of the major enzymes associated with the discolouration of *A. bisporus* tissue (Jolivet, *et al.*, 1995). Tyrosinase belongs to a large group of enzymes classified as oxygenases (oxygenases are enzymes that consume oxygen to oxidize substrates). More specifically, tyrosinase is a polyphenol oxidase (PPO) which is an oxygenase that uses phenolic compounds as substrate for the transfer of oxygen.

PPOs are widely distributed in animals, plants, fungi and bacteria. Mushrooms contain both laccases and tyrosinases. Tyrosinase activity has been found mostly in the fruitbodies, while laccases

are mostly located in mycelia (Aldridge and Walker, 1980). However, of note for future discussions, tyrosinase production by *P. tolaasii* (strains NCPPB741 and NCPPB1116) has been tested (Lelliott, *et al.*, 1966) and shown to be negative.

Physiological function of tyrosinase

The physiological purpose of tyrosinase in mushroom tissue is still unclear because of the complexity of its reaction mechanism, and the formation of products that can be further spontaneously processed into other complicated molecules. Tyrosinase is thought to be a participant in the biosynthetic pathway of 2-hydroxy-4-iminoquinone (Boekelheide, *et al.*, 1980) but has also been attributed to having a role as a defense mechanism because of the properties of its products. o-Quinones can either link proteins and inactivate them, or polymerize into melanin. o-Quinones and melanins have a large bacteriostatic power as they increase the resistance of the cell wall to hydrolytic enzymes (such as glucanase and chitinases) which are secreted by bacteria (Espin, *et al.*, 1997).

Characterization of tyrosinase structure

A. bisporus tyrosinase is one of the most investigated mushroom PPOs. Purification of the enzyme appears complicated and different extraction methods (Strothkamp, *et al.*, 1976, Robb and Gutteridge, 1981, Ingebrigtsen and Flurkey, 1988, Gerritsen, *et al.*, 1994, Papa, *et al.*, 1994, Nunez Delicado, *et al.*, 1996) appear to result in different tyrosinase descriptions (Strothkamp, *et al.*, 1976, Robb and Gutteridge, 1981, Wichers and Leeuwen, 1996).

Tyrosinases from other sources are normally synthesized in a latent form that is activated upon arrival to the designated organelle, normally through cleavage of a peptide by specific proteases. Mushroom tyrosinases are supposed to exist in the cytosol of the cells are lightly bounded to organelles and are in an inactivated form. Commercial mushrooms are white and it is likely that tyrosinase is kept latent as result of a very strong regulation and separation from its substrates by subcellular compartmentalization. Only under certain conditions, such as damage, microbial colonisation, extreme environmental conditions, or aging does it becomes activated and mushrooms discolour.

Tyrosinase activities in mushroom fruitbodies

Tyrosinase activity has been shown to decrease during sporophore development using DOPA experiments (Moore and Flurkey, 1989). Mushrooms that showed similar colour development during growth presented different quantities of tyrosinase and the strain considered of better quality had lower amounts of both the latent and active form of tyrosinase (Leeuwen and Wichers, 1999).

The tyrosinase activity was also investigated during post-harvest development and when stored at different atmospheric conditions. The degree of activation was found to increase during post-harvest development concomitant with the increase of protease activity (Burton, 1988a).

Localization of tyrosinase within mushroom tissue

Visualization of tyrosinase at the tissue level was achieved (Moore, *et al.*, 1988) using vertical and cross-sectional slices of live mushroom blotted onto nitrocellulose. The blotted membranes were stained for tyrosinase activity using DOPA in the presence and absence of tyrosinase inhibitors. Histochemically stained areas of tyrosinase were present throughout the entire tissue, but the gills, stalk and cap epidermis showed darker staining reactions. Furthermore, a study by (Leeuwen and Wichers, 1999) also showed distribution of tyrosinase throughout the mushroom (Figure 1-6).

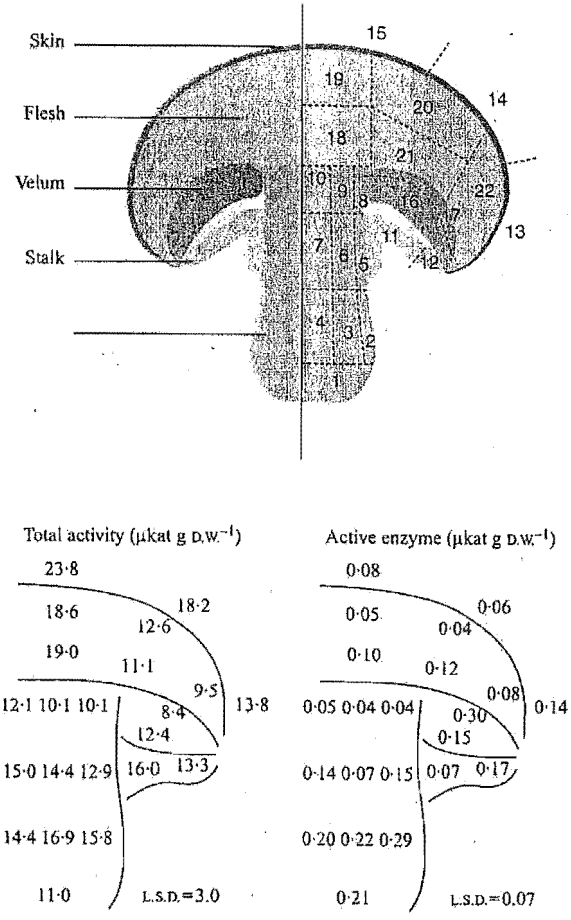

Figure 1-6 Schematic representation of tissues in a *A. bisporus* fruit body and the subdivision used to determine spatial distribution of tyrosinase (Leeuwen and Wichers, 1999).

1.2.3 Overview of *A. bisporus* discolouration processes

Essentially what has been described above is the natural physiological processes which are activated in response to *A. bisporus* cells being damaged by bruising, senescence, or maturation. The other major cause of discolouration is due to microbial interactions. The next section provides a review of literature reporting on selected interaction of microorganisms in the richly biotic environment of mushroom growing.

1.3 Microbial interactions in mushroom growing

Mushroom cultivation, from the composting process to the harvesting of sporophores involves many dynamic interactions between *A. bisporus* and other microorganisms (Stanek, 1974). The environment that *A. bisporus* needs for its development is also a rich source of nutrients and optimal environmental conditions for many other microorganisms. Some of these microbial co-habitants are beneficial for the growth of *A. bisporus*, some have no described effect, and some interactions cause disease and crop losses.

1.3.1 Hyposphere

Microorganisms occurring in the surroundings of mushroom hyphae have been described as "hyposphere microorganisms" and the area in close proximity to hyphae is termed the "Hyphosphere" (Stanek, 1974). The term 'Hyphosphere" was first used to describe a unique environment that provides different environmental conditions for microorganisms than the surrounding compost and casing. This term will be used within this thesis because as has been shown in plant colonising bacteria, the rhizosphere is a very specialised environment promoting the growth of microorganisms with unique phenotypic and morphological adaptations.

1.3.2 Positive microbial involvement in *A. bisporus* cultivation

As discussed, thermophilic fungi are important in the composting process in creating complex carbohydrates for the selective growth of *A. bisporus* (Ross and Harris, 1983, Straatsma, *et al.*, 1989, Op Den Camp, *et al.*, 1990, Wiegant, *et al.*, 1992).

Some bacteria also play an essential role in all facets of mushroom production. They are crucial in composting and initiate sporocarp production. Within the compost, bacteria make available many of the nutrients required by mushroom mycelia and they are also a significant contributor to the compost biomass (Fletcher, *et al.*, 1994). Of the bacterial microflora of the casing layer, over half are pseudomonads, approximately 47% of which are a single species, *Pseudomonas putida* (Samson, *et al.*, 1986). Those bacteria that stimulate rather than inhibit mushroom development (termed 'fruiting potentiators') have also been described including: the first description in 1956 of *Bacillus psilocybe* (Stamets and Chilton, 1983); *P. putida* was isolated and identified as an important potentiator (Hayes, *et al.*, 1969); and then this pseudomonad was the subject of a proposed model for the 'stimulatory effect' on sporocarp initiation and development (Rainey, 1989). Furthermore, three other bacteria (*Bacillus megaterium*, *Arthrobacter terregens* and *Rhizobium metiloff*) and a blue-green alga (*Scenedesmus quadricauda*) have been described as able to stimulate abundant fruitbody formation and development on the soil (Stamets and Chilton, 1983). Some of them are saprophytic such as *P. putida*, which provides *A. bisporus* with the stimulus necessary to 'trigger' basidiome initiation (Hayes, *et al.*, 1969). These and other microorganisms are thought to remove volatile inhibitors of fruit body

initiation, as its effect can be simulated with activated charcoal, which is very effective in adsorbing such compounds (Carlile and Watkinson, 1994). Furthermore, *P. putida* have a close relationship with *A. bisporus* hyphae and has been isolated from internal stipe tissue of healthy mushrooms. This study suggests that because the bacteria are motile and have attachment qualities, they are able to move upward through or between the longitudinally orientated hyphal strands which have aggregated to form the stipe (Zarkower, *et al.*, 1983).

1.3.3 Microorganisms with no described effect

Some microorganisms are also found in mushroom farms that do not exhibit any detrimental effect of *A. bisporus* growth. Bacterial examples that don't elicit any described influence in the growth of *A. bisporus* mycelia include *P. reactans*, *P. marginalis*, and *E. coli* (Preece and Wong, 1982b).

1.4 Pests and diseases of *A. bisporus*

The best described and researched pests and disease of *A. bisporus* is *P. tolaasii* brown blotch however, it is evident that *P. tolaasii* is not the only pathogen of *A. bisporus*. Several other microorganisms described to produce diseases and to reduce the mushroom crop are discussed below. The incidence of these diseases was found to be lower than bacterial blotch but the spoilage of mushrooms is considerable and differs from one country to another. Diseases of *A. bisporus* can essentially be divided into major groupings of causative organisms: fungi (moulds and diseases), viruses, insect pests and pathogenic bacteria (Fletcher, *et al.*, 1994).

1.4.1 Moulds in mushroom cultivation

'Moulds' are defined as fungi other than the cultivated mushroom and its fungal pathogens that occur in compost or casing at any stage of cropping (Fletcher, *et al.*, 1994). Moulds are often reported in mushroom growing and their presence is a signal that the compost has not been properly prepared and such practices as proper peak heating and cooking out have not been adhered to. Therefore, moulds are often described as 'indicators', *i.e.* their presence indicates improper farming practices (Geels, *et al.*, 1988). Moulds are generally considered to be competitors for nutrients, or antagonists of *A. bisporus* rather than parasites. Weed moulds such as *Acremonium murorum*, *Penicillium* sp., *Trichoderma atroviride* and *T. pseudokoningii* can affect mushroom yields of *A. bisporus* by 8-94% (Grogan, *et al.*, 2000). However, because the presence of weed moulds usually indicates improper farm management and hygiene, more significant disease and pests will establish.

1.4.2 Fungal diseases

Fungal diseases are a very important group of pathogens to cultivated mushrooms. Fungal diseases will vary from incidence to severity over time, but are invariably always present because they are well

adapted to survival and persistence in mushroom farms and have the evolutionary ability to develop resistance to chemical fungicides. Fungal pathogens are covered in this literature review not only because of their prominence within the mushroom industry, but also because many fungal pathogens have early disease phenotypes that resemble bacterial blotch and therefore many mushroom growers often misidentify disease (Figure 1-7).

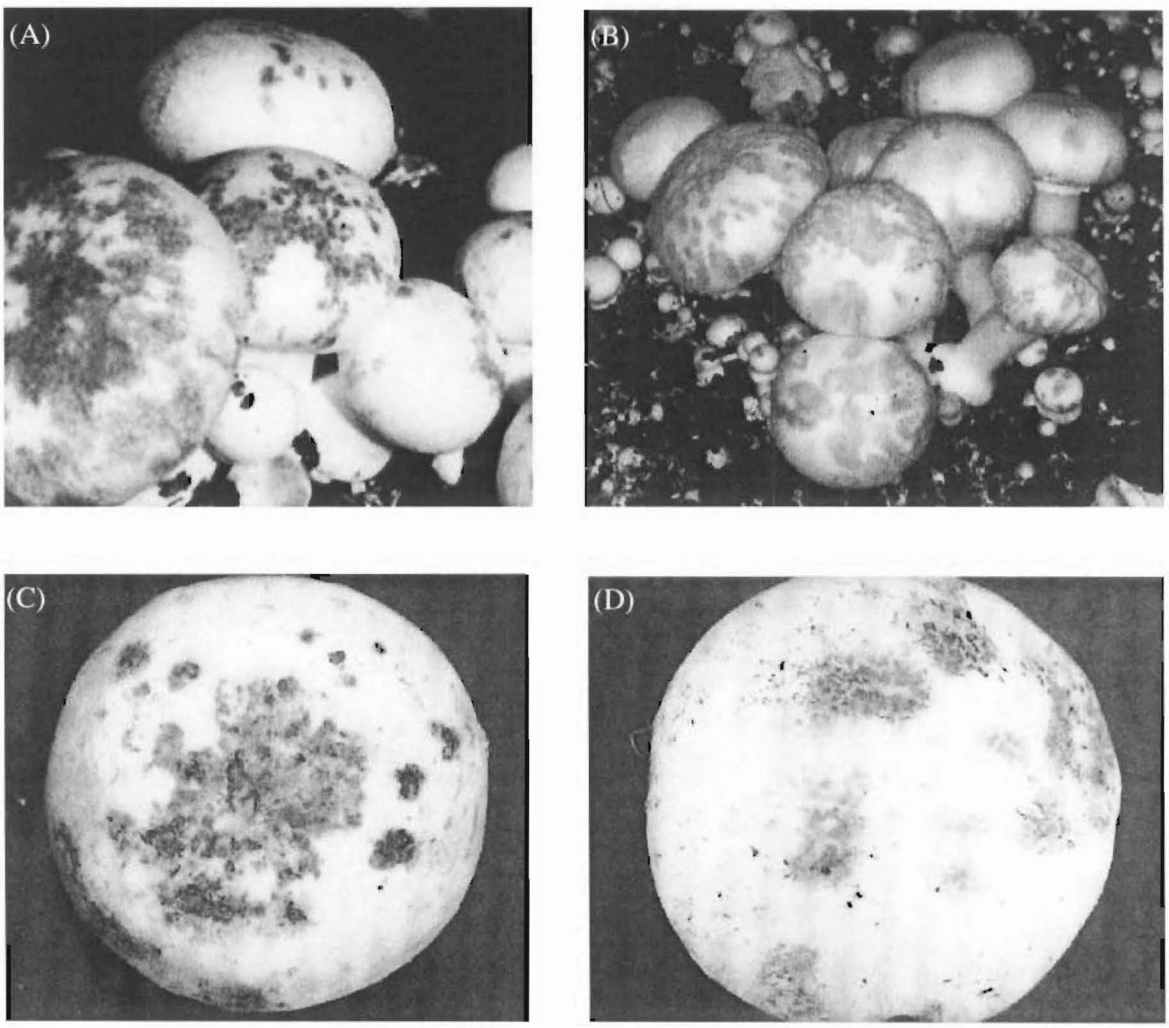

Figure 1-7 Comparison of cap spotting caused by different diseases: A) bacterial blotch caused by *P. tolaasii*; B) *Verticillium fungicola* spotting; C) *Dactylium* spotting; and (D) *Trichoderma* spotting.

Verticillium dry bubble disease

Verticillium fungicola (also called *V. malthouse*) and *V. psalliotae* (Chen, *et al.*, 1981) are described as causing dry bubble disease in *A. bisporus* (Gandy, 1979). They are endemic fungi from the casing, especially the peat (Wong and Preece, 1987). This disease is world wide in distribution (Collopy, *et al.*, 2001) and seems to be the second most important disease of *A. bisporus* after *P.*

tolaasii in causing loss of crops for the growers (Zaayen, 1982). *V. fungicola* is a true parasite of *A. bisporus*, deriving its nutrition from the host following intra-hyphal penetration of mushroom mycelium (Draght, *et al.*, 1996). Slightly diseased mushrooms are characterized by brown coloured spots or streaks on the basal or upper regions of the stem and on the caps of developing primordia. These spots later become grayish coloured from spore production. If the mushrooms develop with *Verticillium* infection, they are grossly malformed with young primordia being turned into sclerotia-like balls of amorphous whitish mycelia (Stamets and Chilton, 1983).

No *Verticillium* toxin has been detected so far but *V. fungicola* possess extracellular cellulases, laccases (Rai, *et al.*, 1993) and an extracellular protease (Kalberer, 1984) that can cause degradation of the hyphae resulting in browning. The protease enzyme has been suggested to be responsible for the activation of mushroom tyrosinase in *Verticillium* infected mushrooms (Thapa and Jandaik, 1989). Many fungicides and mixtures of chemicals have been tested to control *Verticillium* (discussed in (Gea, *et al.*, 1996)), but none have been completely successful. However, new strains of *V. fungicola* resistant to those fungicides have been isolated (Bollen and Zaayen, 1975, Gea, *et al.*, 1996, Bonnen and Hopkins, 1997) mainly due to incorrect chemical dosage application and continual use of a single fungicide. As well as for *P. tolaasii*, other types of control have been tested, such as to treat the casing with heat aerated steam (Moore and Wuest, 1973) and biological control using *Trichoderma viride* (Trogoff and Richard, 1976). However, with *Verticillium* and all other diseases described below, as well as inhibiting the development of the disease, it is equally important to implement farm management strategies to prevent spreading of the disease to other growing rooms or other farms (Geels, *et al.*, 1988).

***Dactylium dendroides* disease**

Dactylium dendroides (also known as *Cladobotryum dendroides*) causes the disease known to mushroom growers as 'cobweb mould', thus called because of coarse mycelia covering affected mushrooms that looks like cobwebs (Fletcher, *et al.*, 1994). *Dactylium* is a soil-inhabiting fungus, which may be introduced into mushroom casing as spores or mycelium fragments. Conidia, which cause cap spotting and the establishment of further colonies, are vital to the dispersal and persistence of the disease (Dar, 1997). The cap spotting caused by *Dactylium* spotting results in spots that are often mistaken for bacterial blotch. *Dactylium* is best recognised when the conidia form the 'cobweb' patches of mycelia that rapidly colonize the casing surface and eventually causing affected mushrooms to turn brown and rot. Fungicide resistance is also a concern in *Dactylium* and isolates are being identified that show fungicide resistance (McKay, *et al.*, 1998, Grogan and Gaze, 2000). Further to this, *Dactylium* is a major problem in that the spores are resistant to desiccation and may remain in soil surrounding the farm for up to six months, resulting in continual re-contamination of a mushroom farm.

Trichoderma blotch disease

Several *Trichoderma* strains (*Trichoderma harzianum, T. viride, T. atroviride, T. aggressivum* f. *aggressivum* (Samuels, *et al.*, 2002)) often are parasitic toward *A. bisporus* by inhibiting cultivation or reducing the fruitbody production. *Trichoderma* is encouraged by improperly adjusted environmental parameters, such as excessively wet casings applied to sterile grain spawn, high and prolonged humidity in combination with stagnant air and high carbon dioxide levels. *Trichoderma* frequently grows on the wooden trays holding compost and also on the casing soil with peat or humus. Parasitised mushrooms have dry brownish blotches or sunken lesions on the cap or stem. They are often enveloped by a fine downy mildew that may eventually become greenish from spore production, and are grossly disproportioned. A fuzzy mycelia may be present on the cap. These lesions are dry, whereas the blotches caused by bacteria tend to be moist (Stamets and Chilton, 1983). *Trichoderma* is alleged to secrete toxins that inhibit *A. bisporus* primordia formation and growth (Stamets and Chilton, 1983).

Trichoderma harzianum Rifai is described to exist in one of three biological forms (Muthumeenakshi, *et al.*, 1994) and group 2 (Th2) is the main agent responsible for green mould epidemics affecting *A. bisporus* in the British Isles (Seaby, 1987). Th2 was found to have an enhanced colonisation rate (Sharma, *et al.*, 1999) and produce volatile metabolites *in vitro* that had fungistatic effects on the growth of *A. bisporus* (Mumpuni, *et al.*, 1998). The occurrence of *Trichoderma* was also reduced if the peak heat in the composting was extended in temperature or time (Park, *et al.*, 1971), again indicating the importance of proper farm management.

Other fungal diseases

The wet bubble disease (Stamets and Chilton, 1983), shaggy stipe disease (Fletcher, 1973), *Gliocladium deliquescens* spp. (Thapa and Seth, 1989) and *Pythium oligandrum* (Godfrey, *et al.*, 2003) are also examples of the wide variety of fungal infections that can occur during all the steps of the mushroom cultivation. Economically, these diseases are not important, despite causing crop yield reduction but are normally easier to control by proper monitoring of environmental conditions, especially peak heating.

1.4.3 Viral diseases

Mushrooms can provide a host for viral particles (Nair, 1972, Nair, 1973, Last, *et al.*, 1974, Revill, *et al.*, 1994). Although healthy mushrooms have double-stranded RNA (dsRNA) bands (Romaine and Schlagnhaufer, 1989), new virus particles have been identified in mushrooms that cause disease. The two major virus diseases reported are La France and Virus X.

La France

La France disease (also known as dieback, watery stipe, and brown disease) is amongst the most serious infectious pathologies of *A. bisporus* (Ghabrial, 1994). La France disease is considered to be a

double-stranded-RNA (dsRNA)-genome isomeric virus (Goodin, *et al.*, 1992) and mushrooms and mycelial cultures manifesting the disease symptoms contain nine virion-associated dsRNAs (0.8 to 3.8 kb) (Koons, *et al.*, 1989). La France disease symptoms include slow and aberrant mycelial growth, reduced yield and the development of malformed mushrooms displaying premature maturation and accelerated postharvest deterioration. Because of the lack of effective resistance within *A. bisporus*, management of the disease is limited to the performance of hygienic practices directed at the elimination of infected fungal propagules from the production areas (Schisler, *et al.*, 1967). Today, established molecular diagnostic techniques using reverse transcription PCR analysis (Romaine and Schlagnhaufer, 1995) of regularly provided samples ensures monitoring of La France disease.

Virus X

A serious disease phenomenon affected the British mushroom industry and was first observed in 1996. Since this time several other farms had described severe crop losses, with some farm closures (Gaze, 1999). The symptoms of this new disorder have been quite diverse (Gaze, *et al.*, 2000). Areas of non-productive beds varying in size from small patches within normal cropping surfaces to, in extreme cases, being large areas of non-growth. These patches gave rise to the original name of 'patch disease'. However, molecular analysis of affected mushrooms showed that the disease is caused by a novel dsRNA virus or viruses termed 'Virus X' (Gaze, *et al.*, 2000). Development of RT-PCR analysis of Virus-X is currently under development at Horticulture Research International (Bruce Adie, pers. comm.). Otherwise, the disease is managed as for La France disease, farm management with a focus on hygiene practices directed at the elimination of infected fungal propagules from production areas.

1.4.4 Insect pests

Apart from the direct involvement in mushroom yield reduction described above, the major role in which these insects play is that of a vector for disease transmission of microorganisms. Fungal spores and bacterial cells readily adhere to nematodes, mites and flies that then transfer the disease around the mushroom farm (Wong and Preece, 1980, Grewal and Wright, 1992).

Flies

Insect pests causative of many problems are various species of *Diptera* (flies) that includes the two most prominent types, Sciarids (*Lycoriella solani* and *L. auripila*) and Phorids (*Megaselia halterata* and *M. nigra*) (Fletcher, *et al.*, 1994). Flies are attracted to the mushroom crop at various production stages and it is the larvae which cause the damage by feeding directly on the mycelia, swarming on sporophores, or tunneling into the developing or developed sporophores. Therefore symptoms of flies include reduction of yield due to loss of mycelia and direct damage of the mushroom.

Mites

Mites also feed on mushroom mycelia and on the developed mushrooms, where they cause surface discoloration and reduction of yields (Wu and Yu, 1996).

Nematodes

Nematodes are often present on mushroom farms (Grewal and Wright, 1992) and become visible when they aggregate into clusters on the casing surface. Some species of nematodes feed on the mushroom mycelia directly (mycophagous nematodes) and reduce yield although most feed on decaying matter (saprophagous nematodes). The presence of nematodes is mostly a sign of inefficient peak heat practices (Fletcher, *et al.*, 1994).

1.4.5 Bacterial diseases

Many bacterial diseases associated with mushroom cultivation have been described and are reviewed below. Although bacterial spoilage has not been reported to cause detriment to human health, bacterial diseases are so prominent because they can result in large economic losses due to low aesthetic qualities that reduce consumer appeal in the market place.

Dripping gills and yellow blotch disease

P. agarici have been described as causing drippy gill and yellow blotch. Drippy gill disease (Young, 1970) has been identified worldwide, including New Zealand (Gill, 1994), Korea (Jin, *et al.*, 1994), India (Jandaik, *et al.*, 1993), The Netherlands (Geels, *et al.*, 1994) and the UK (O'Riordain, 1972a). This disease is characterized by pale brown discolouration, after only 24 h of infection, mushrooms showed a brownish marbled appearance. Only the outside of the cap is affected and no further abnormalities are observed. The affected area feels sticky and the superficial tissue layer of affected cap can easily be removed by gently rubbing the surface of the cap (Geels, *et al.*, 1994). The yellow blotch disease seems to occur if a very high humidity is used or excessive watering of the bed before pins appear, combined with a high temperature occurring in the houses. It does not cause as much loss as *P. tolaasii* (O'Riordain, 1972c). Sometimes the disease has been present but unnoticed, in particular if the mushrooms are picked as buttons (O'Riordain, 1972b). Oxytetracycline, streptocycline (Jandaik, *et al.*, 1993) and a low concentration of sodium hypochlorite (O'Riordain, 1972b) seem to effectively reduce the disease.

Ewingella americana

Ewingella americana was isolated from *A. bisporus* mushrooms exhibiting internal stipe necrosis in the United Kingdom (Inglis, *et al.*, 1996). Furthermore, it was shown that *E. americana* exhibits constitutive synthesis of chitinolytic activity of a endochitinase of 33-kDa (Inglis and Peberdy, 1997).

1.4.6 Blotch diseases

Pseudomonads originating from the casing layer are regarded as causative of the majority of described bacterial diseases (Fletcher, *et al.*, 1994). Discolouration of *A. bisporus* caused by pathogenic pseudomonads, the so called 'blotch diseases', are well documented. In essence, mushrooms promote an optimal niche for bacteria because they are composed of many nutrients (Chapter 1.1.4). If excess moisture is present on the mushroom cap, nutrients will leak from the mushroom into this water film and provide a nutritional rich environment in which bacteria will exponentially multiply (Geels, *et al.*, 1988). Bacteria possessing pathogenicity determinants will cause blotch disease symptoms. Three major bacteria able to cause varying blotch discolouration of *A. bisporus* mushroom tissue have been described in the literature: *P. gingeri* causes pale yellowish-red discolourations that develop into reddish-ginger coloured discolouration (ginger blotch disease) (Wong, *et al.*, 1982); *P. reactans* causes mild dark-purple to light-brown discolouration and a slight surface depression that becomes deeper and darker with age (Wells, *et al.*, 1996); and *P. tolaasii* results in sunken, dark brown lesions (Tolaas, 1915, Paine, 1919).

Ginger blotch disease (*P. gingeri*)

Ginger blotch was reported in some UK mushroom farms from 1977 onward and then later in The Netherlands, France (Guillaumes, *et al.*, 1985), Australia (Cutri, *et al.*, 1984), and New Zealand (Gill Hoglund pers. comm.). The symptoms seemed to differ from those described for the brown blotch and in 1982, Wong *et al.* discovered that ginger blotch was caused by a previously undescribed bacterium. This bacterium was later named *P. 'gingeri'* (Preece and Wong, 1982b) but its name has not yet been officially validated in bacterial nomenclature.

P. gingeri initially produces small pale Yellowish-red-brown flecks which cover 20 - 25 % of the surface of young sporophores. Usually the lesions developed round the edges of the affected caps, often affecting their entire circumference. Then, when the infection becomes more severe, reddish-ginger coloured spots covering the entire mushroom surface appear (Wong, *et al.*, 1982). The colour of the lesions is easily distinguishable from the chocolate-brown lesions of *P. tolaasii* blotch. The ginger discolouration was observed to be superficial, extending 1-2 mm only below the mushroom cap surface. Most affected sporophores did not show distortion of the cap, and the lesions were not noticeably sunken. Occasionally in later flushes some cracking and shallow pitting effects were observed in the lesions and ginger-blotched mushrooms were usually slimy upon touching. It appeared to be most severe in the summer months and the worst outbreaks were observed when the air humidity was high (90%) in the cropping houses. The bacterium, as *P. tolaasii*, reduced also the yield of mushrooms per bed. Some mushroom caps were observed to present both *P. gingeri* and *P. tolaasii* spots. *P. gingeri* can be distinguished from *P. tolaasii* because its colonies are mucoid (gray-green) on PAF, do not produce a positive white line test, or mushroom tissue pitting, (Wong and Preece, 1979). Additionally, the opposite response to 2-ketogluconate, lipase (Wong, *et al.*, 1982) and lecithinase

(Cutri, *et al.*, 1984) tests can be used for discrimination between *P. gingeri* and *P. tolaasii*. *P. gingeri* can be also recognized because it produces a unique acidic EPS (Cescutti, *et al.*, 1995, Fett, *et al.*, 1995).

P. gingeri can be expressed in two relatively stable phenotypic forms. As well as *P. tolaasii*, the smooth pathotype is pathogenic and the rough pathotype is non-pathogenic. The severity of the disease can be due to the number of pathogenic forms mixed with non- pathogenic forms in the infected cap (Cutri, *et al.*, 1984).

P. reactans

P. reactans is a strain that is considered closely related to both *P. tolaasii* and *P. gingeri* because of similarity of environmental niche and biochemical phenotypic characteristics. *P. reactans* is best described in the literature as the indicator organism that provides an *in vitro* biochemical test called the white-line-in-agar assay (WLA) for the identification of *P. tolaasii*[1]. Like *P. tolaasii* and *P. gingeri*, *P. reactans* has been identified in pathogenic and non-pathogenic forms. The pathogenic state is responsible for small, dark purple spots on harvested mushrooms (Wells, *et al.*, 1996), but description of disease caused by *P. reactans* is sparse. The non-pathogenic form, saprophytic on the mushrooms, has also been described as an antagonist of *P. tolaasii* (Grewal and Rainey, 1991, Soler-Rivas, *et al.*, 1999b), surmised to be because it competes for nutrients.

***P. tolaasii* Brown Blotch**

Of the bacterial blotches, brown blotch caused by *P. tolaasii* is the best characterised in current literature. Therefore, the following sections contain a detailed overview of brown blotch caused by *P. tolaasii*. Please note that a further review on the physiological and genetic determinants of *P. tolaasii* brown blotch is included in Chapter 2, with a subsequent focus in Chapter 3 on the extracellular toxins produced by *P. tolaasii* during brown blotch disease.

1.5 Brown blotch disease caused by *P. tolaasii*

1.5.1 The incidence of brown blotch internationally

The incidence of the blotch disease in various countries is different and changes from year to year. In France, where cultivation is carried out mostly in caves, the incidence of blotch has been reported to vary from 8 to 50% of the crop (Olivier, *et al.*, 1978), approximately 10% of United Kingdom crops is rendered unmarketable (Upstone and Carter, 1979) and a further 10% of the crop is downgraded as a result of infection (Rainey, *et al.*, 1992). In Italy, crops have been observed to be reduced up to 40% in dramatic years (Ercolani, 1970) and in The Netherlands, 10-15% of the production is affected,

[1] The WLA is described in detail in Chapter 2.6.5.

which means that more than 34500 tonnes are discarded every year, causing a very important economic loss (Janse, *et al.*, 1992).

Other continents with an important button mushroom market are also seriously affected, such as the USA (Tolaas, 1915), Australia (Nair, 1975, Fahy, *et al.*, 1981), Israel (Bashan and Okon, 1981) and Turkey (Ozaktan and Bora, 1994). In some other countries, where the button mushroom production is less important than the cultivation of other mushrooms species, bacterial blotch was also described as an important problem: Japan (Suyama and Fujii, 1993), Korea (Kim, *et al.*, 1995), China (Cutri, *et al.*, 1984) and India (Guleria, 1976).

1.5.2 *P. tolaasii* causes brown blotch

P. tolaasii is easily able to colonise mushroom farms given the non-aseptic conditions in which compost and casing materials are prepared. Once present on a farm, *P. tolaasii* has the ability to form unattractive brown or cream spots on developing mushrooms ca*P.* In severe cases, they become dark brown and sunken and can cover the entire mushroom cap surface. The yield decreases and the quality is downgraded. *P. tolaasii* is endemic of the compost and casing soil cohabiting with other *Pseudomonas* strains beneficial for the mushroom. *A. bisporus* hyphae excrete compounds able to induce chemotaxis of *P. tolaasii* and once the bacteria detect the presence of a nutrient source such as *A. bisporus* mycelium, they migrate towards it, reach the surface of the hyphae, and attach. *Pseudomonas* strains develop filament-like structures that are morphologically different from flagella, fimbriae or pili and appear to connect the bacteria to the mycelial surfaces and to each other. They also excrete a large amount of polysaccharides, thereby fixing themselves firmly to the hyphal surface. Subsequently, the bacterium can provoke the permeation of the membranes through tolaasin action making the nutritive content of the cytoplasm accessible for the bacterial growth.

Artificial inoculations of mushroom compost beds indicate that very small concentrations of bacteria are able to initiate the disease and to give rise to severe epidemics. A method, assessing mushroom blotch resistance, reveals that no mushroom strain is completely resistant to the disease. A great diversity in the intensity of the symptoms is observed among a wide range of commercial and wild strains tested. Mushrooms are known to produce extracellular bactericidal enzymes and scanning electron microscopy images indicate that *A. bisporus* produces electron dense material as a defense barrier, but the nature of this compound is still unknown. Several methods have been tested to control the incidence of the pathogen. High relative humidity and temperature were reported as optimal environmental conditions for the development of the disease, due to the water condensation occurring on the ca*P.* Treatment of the mushroom beds with chemicals such as sodium hypochlorite, chloride derivatives, bronopol and antibiotics reduced the symptoms of the disease. However, the administration of the chemical by the growers can be complicated and does not prevent completely the symptoms of the disease. Biocontrol using antagonists and bacteriophages also reduced the incidence of the pathogen, but not completely.

1.5.3 Symptoms

Brown blotch disease of the cultivated mushroom was first described by Tolaas in 1915. The causal agent, *P. tolaasii*, was later identified by Paine (1919). Colonization of mushroom caps by the bacterium results in development of unappealing brown or cream lesions on the pileus and stipe. These lesions are slightly concave spots, round or spreading in many directions (Olivier, *et al.*, 1978). The spots may be small (1-4 mm diameter) and pale brown but in more severe cases they are dark and sunken. The browning affects only the external layers of the cap tissues, and is restricted to 2-3 mm below the surface of the cap (Tolaas, 1915). The lesions may coalesce to cover the entire mushroom surface although only small black or brown punctuation's are sometimes observed (Rainey, *et al.*, 1992).

When a farm is affected, the first indication of the infection is a fairly late onset of fruitbody formation and reduction of the crop, where maturing or mature mushrooms (all developmental stages) develop superficial brown lesions. Blotch occurrence can vary from small lesions occurring on a few mushrooms, to a severe incidence where all the mushrooms on a bed will be severely affected. It can be noticed since the early first flush until the latest (Miller and Spear, 1995).

Some mushrooms seem to be healthy after picking but later during postharvest storage they develop symptoms. This may even occur when stored at low temperatures, because the bacterium possesses the ability of growing at low temperatures (Nair and Bradley, 1980). In harvested mushrooms a faster rate of deterioration was observed, in particular when they are stored in conditions of high relative humidity such as in film over wrapped packages (Beelman, *et al.*, 1989). The brown spots cause mushrooms to be also refused as high quality produce as well as being rejected in canning factories.

1.5.4 Sources of disease in the mushroom farms

Nair and Fahy (1973) ware the first authors who drew attention to the casing layer as a potential source of pathogenic pseudomonads, but they failed to isolate these pathogens from peat, and did not test the limestone. Later, Wong and Preece (1980) carried out an extensive search for *P. tolaasii* in a commercial farm. All the materials, machinery, workers, houses and other items related to the various processes of mushroom production, as well as different flushes, spores and pests were qualitatively studied looking for the primary and secondary sources of the pathogenic *P. tolaasii*. The incidence of the pathogen in the component of the casing soil was low, but since no significant amount of bacteria were isolated from farm soil, water, spawn or spawned compost before casing, the peat and limestone were identified as primary sources of the pathogen in clean houses. Once the pathogen is in the farm, the secondary source of *P. tolaasii* infection is spread by pests and on picker hands that transfer the bacterium from one house to another.

More detailed studies on several composts used in various farms appeared to differ from those findings (Fahy, 1981). The pathogen was present in compost before and after peak heating, during spawning and during cropping. Peat from casing onwards maintained a high level of the pathogen even in crops without symptoms of the disease. The pathogen was also present in healthy crops in the casing at population densities equal to crops with a high incidence of the disease. Thereby, the primary source is the compost and the casing material required for the mushroom cultivation. The disease organisms can therefore be regarded as being 'ever present'.

In compost, fluorescent pseudomonads are very common representing 10 % of total aerobic population that was evaluated as 10^7 - 10^8 bacteria per gram of compost. Generally they rose up to 50 % - 90 % of a total of 10^9 bacteria, 4 to 10 days after spawning and stayed approximately at that level, or decreased slowly till the first flush (Samson, *et al.*, 1986). The casing soils contained generally fewer bacteria than compost (an average of 6 x 10^6 bacteria per gram). *Pseudomonas* is an endemic genus from peat and limestone that includes numerous pathogenic strains that react positively in the white line test (Wong and Preece, 1979) with *P. reactans* and saprophytic pseudomonads highly important for mushrooms. Migration of *P. tolaasii* from compost into the casing soil was also reported (Samson, *et al.*, 1986). Most of the pseudomonads identified were *P. fluorescens* or *P. putida*. The heterogeneous group of *P. fluorescens* represented 33 % and 31.5 % of the pseudomonads, from compost and casing soils respectively. Some of them belonged to biovars I, II and III but the majority of them were classified as biovar V (including *P. tolaasii*). *P. putida* represented 56 % of the microbial population in composts and 47 % in casing soils (Samson, *et al.*, 1986) and a stable equilibrium between *P. fluorescens* and *P. putida* was observed. An increase of the *P. putida* populations during the mycelial development of the mushroom and a relative decrease of *P. fluorescens* was noticed directly related to the speed of mycelia growth. The saprophytic *P. putida* was found essential for crop production because it induces sporophore formation in *A. bisporus* (Hayes, *et al.*, 1969).

1.5.5 Abiotic conditions that favour blotch formation

If, after watering, mushroom caps are not dry within 2-3 hours, or if changing temperatures give rise to condensation on the mushroom caps which is not evaporated within two to three hours, the conditions are created for blotch causing bacteria to multiply (Geels, *et al.*, 1988). Water provides a medium in which nutrients from mushroom tissue leaches nutrients into the cap moisture providing a 'soup' for bacterial growth to occur.

The effects of environmental factors on increases in numbers of the pathogen and on symptom development differ in various reports. Gandy (1967) claimed that a RH of 82% was favourable to the production of blotch and the disease was dramatically reduced dramatically if the RH was maintained at 75%. Overhead watering of existing blotch increased the disease severity. Sinden (1971) (Sinden, 1971) found that fluctuation of temperature as little as 0.5 °C was enough to induce blotch disease. He

also reported that blotch disease could be initiated if overhead splashing of water on pin size primordia took place. Nair and Bradley (1980) observed that all mushrooms in all farms carry a population of the blotch bacterium. It was, therefore, a potential time bomb and the fuse was the surface wetness. (Preece and Wong, 1982a) reported that neither raising the temperature from 16 to 19°C, nor increasing the air RH from 70 - 90 % significantly affected bacterial numbers or disease incidence. Overhead watering, onto the upper surface of the caps, increases both numbers of *P. tolaasii* and also disease levels, but has to be excessive (every other day) to do so. There appears to be enough water available to the pathogen within the mushroom itself for disease development.

The above mentioned reports elude to the fact that abiotic conditions contribute heavily to the development of bacterial blotch. Furthermore, personal communication with mushroom growers throughout New Zealand, Australia and the United Kingdom confirm that excessive moisture on the mushroom cap that is not removed by evaporation will result in the development of blotch lesions.

1.5.6 Characteristics and quantification of bacterial thresholds for blotch

Artificial inoculation of *P. tolaasii* in the casing material within commercial mushroom beds was performed to investigate the bacterial concentration and the symptom development in controlled environments (Preece and Wong, 1982b). Very low numbers of cells of *P. tolaasii* (20 cfu.cm^2) present in a casing surface can give rise to severe epidemics of bacterial blotch disease, as the bacterium multiplies on the fungal mycelia and on developing mushrooms. Different concentrations have been reported as threshold values, ranging from 6 x 10^7 (Preece and Wong, 1982b) to 1 x 10^5 (Nair and Bradley, 1980) cells per cap. 10^7 to 10^9 cfu.ml^{-1} (Olivier, *et al.*, 1997) (2 x 10^5 to 2 x 10^{10} cells per cap), or 7.7 x 10^4 (Nair and Fahy, 1972a) to 10^9 cfu.cm^2 (Rainey, *et al.*, 1992).

The threshold value was found to be irrespective of size or age of mushroom cap and the disease may be visible in crops long before it has been detected hitherto. Many mushrooms have been identified with heavy infection by *P. tolaasii* (5.4 x 10^6 detectable cells) but exhibit no obvious symptoms, and are thus 'carriers' of the pathogen (Preece and Wong, 1982a).

1.5.7 Bacterial colonisation of *A. bisporus* hyphae

The high number of bacteria in the vicinity of fungal hyphae (the hyphosphere) can be attributed to an increased availability of nutrients provided by the mycelia that can act as metabolite stimulants and nutrients for the bacteria. Materials released from the hyphosphere into the surrounding water particles may contain amino acids, proteins, sugars, complex carbohydrates, enzymes, organic acids, and vitamins.

Chemotaxis

Chemotaxis is a common attribute of many bacteria and is broadly defined as the ability of a motile cell to respond to chemical environmental signals by altering its pattern of motility. Chemotaxis

enables bacteria a means to locate nutrients and avoid harmful environments (Chet and Mitchell, 1976). Chemotaxis is also important in microbial interactions and colonisation by bacterial cells onto their host (de Wager, *et al.*, 1987) and has been observed to be important for bacterial/fungal interactions (Arora, *et al.*, 1983, Lim and Lockwood, 1988). Hyphal and spore exudates have been shown to provide growth substrates for bacteria. In the porous, water-saturated, nutrient-poor casing layer, leakage of hyphal contents by *A. bisporus*, and/or production of metabolites, would provide a nutrient gradient with ideal conditions for bacterial migration (Samson, *et al.*, 1986). *A. bisporus* mycelia produces compounds which can be used by pseudomonads to grow, in particular by *P. putida* (Hayes, *et al.*, 1969). *P. tolaasii* is also a motile bacterium and it can modify its position by chemotactic attraction toward 'signals' produced by the *A. bisporus* mycelium. The ability to move toward these nutrient sources may provide the bacteria with a distinct survival advantage and may also aid colonization of individual mycelium.

In the presence of *A. bisporus* exudate (max. 0.01% (w/v)), *P. tolaasii* as well as *P. putida* display a marked positive response, but the nature of the attractants is still unknown (Rainey, 1991). *P. tolaasii* showed a positive response towards casamino acids (maximum response to 0.1 % (w/v)). No response was found towards sugars (Glucose, mannose and rhamnose) and very little towards single amino acids. Heat treatment and dialysis of an *A. bisporus* exudate revealed that low molecular weight thermostable compounds are a source of attractants, but the unidentified chemo-attractant(s) may be a mixture of compounds. The spontaneous phenotypic variant[1] showed a more rapid chemotactic response than the wild-type form (time- and dose-dependent). This ability of the phenotypic variants may confer upon them an additional advantage and is consistent with the hypothesis that these forms are more suited to survival under nutrient-stressed conditions, where the ability to locate new and favourable niches is of prime importance (Rainey, 1991). The slower chemotactic response of the original rough variants suggests they may not survive so well under nutrient-depleted conditions. Instead, they may be suited to nutrient-rich environments that may be provided by the *A. bisporus* mycelium when nutrients are liberated by the membrane disruption by the extracellular toxin, tolaasin[2] (Rainey, *et al.*, 1991).

Pleurotus ostreatus is another mushroom host for *P. tolaasii* and was found to also produce compounds from its mycelia that could attract chemotactically the pathogen and even stimulate the toxin production (Murata and Magae, 1996). By contrast to *A. bisporus*, the activating molecules in *P. ostreatus* could be related to glucose, glycerol or trehalose which appear to act as signals that *P. tolaasii* can detect *in vitro*. At present, the exact nature of the primary substance is unclear.

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[1] Colony transition is discussed in Chapter 2.4

[2] Tolaasin is extensively reviewed in Chapter 3.3 in respect to its membrane disruption of *A. bisporus* cells.

Attachment

Once the bacteria sense the presence of a nutrient source such as *A. bisporus* mycelia, they migrate towards it and reach the surface of the hyphae. Adherence of bacteria to surfaces of living organisms can be an important factor in determining the extent to which bacteria influence the growth and development of a host (Schroth and Hancock, 1982, de Wager, *et al.*, 1987). Direct contact of *P. putida* with hyphae is necessary for basidiome initiation to occur (Hayes, *et al.*, 1969) and colonization of mycelial surfaces by *P. tolaasii* is thought to be important in relation to the development of brown blotch disease (Preece and Wong, 1982b). Wild-type *P. tolaasii* was found consistently attached to the mycelium, and in greater numbers than the phenotypic variant (Rainey, 1991). 99.3 to 84 % of the cells were strongly attached immediately after inoculation (Preece and Wong, 1982a), whereas smooth cells was attached after 10 min. These could not be removed with a water rinse of the mycelium, while only a few cells of the phenotypic variant of *P. tolaasii* were still attached (Preece and Wong, 1982b). Therefore, the attachment process, in quantitative terms, differed markedly between pathogens and non pathogens.

P. tolaasii as well as *P. putida* were relatively hydrophilic so the attachment did not depend on hydrophobic interactions. The presence of polysaccharide polymers and fibrillar appendages on the surface of *P. tolaasii* may be responsible for attachment. Such appendages may enable bacteria to overcome the repulsion energy barrier which exists when two negatively charged surfaces are brought in close proximity and achieve rapid, firm attachment to the hyphal surface (Rainey, 1991).

Pseudomonas strains were compared by SEM on the *A. bisporus* hyphae. *P. tolaasii* had the highest percentage detectable attachment (DA) of the bacterial species tested (73.8), followed by *P. gingeri* (65.0), *P. putida* (31.0), *P. reactans* (30.6) and *P. marginalis* (18.9). The percentage DA was found correlated to the pathogenicity, distinguishing between pathogenic and non-pathogenic bacteria (Preece and Wong, 1982b).

1.5.8 Microscopic studies of *P. tolaasii* / *A. bisporus* interactions

Types of *A. bisporus* hyphae

Healthy *A. bisporus* mycelia growing in the compost and the casing layer were observed under scanning electron microscopy (SEM). The mycelia consisted mostly of thin (1 to 2 mm wide) single hypha with calcium oxalate crystals surrounding them. This type of vegetative hyphae is typical of the fungus growing in compost (Cochet, *et al.*, 1992).

Bacterial populations attached to the *A. bisporus* hyphae by SEM

Observation of the bacteria associated with hyphae in the inoculated peat revealed both rods and cocci shaped bacteria (Eicker, *et al.*, 1989). Some bacteria were very close to the hyphae, and some were attached to hyphae and to each other by filament-like structures that were morphologically different from flagella, fimbriae, or pili. These structures were observed on washed hyphae in

association with attached rod-like bacteria which appeared alone, in pairs, or in aggregates on the hyphal surface (Masaphy, *et al.*, 1987) and appeared to join the bacteria both to the mycelial surfaces and to each other (Rainey, 1991).

Most individual bacterial cells lay flat against the hyphae, with a few attached by one end of the bacterium only. There appeared to be no difference in the arrangement or mode of attachment of the bacterial cells to the hyphae 30 min after washing with distilled water. In all cases, the attached bacteria were apparently joined to the hyphal surface and to each other by the rod-like connections (Preece and Wong, 1982b).

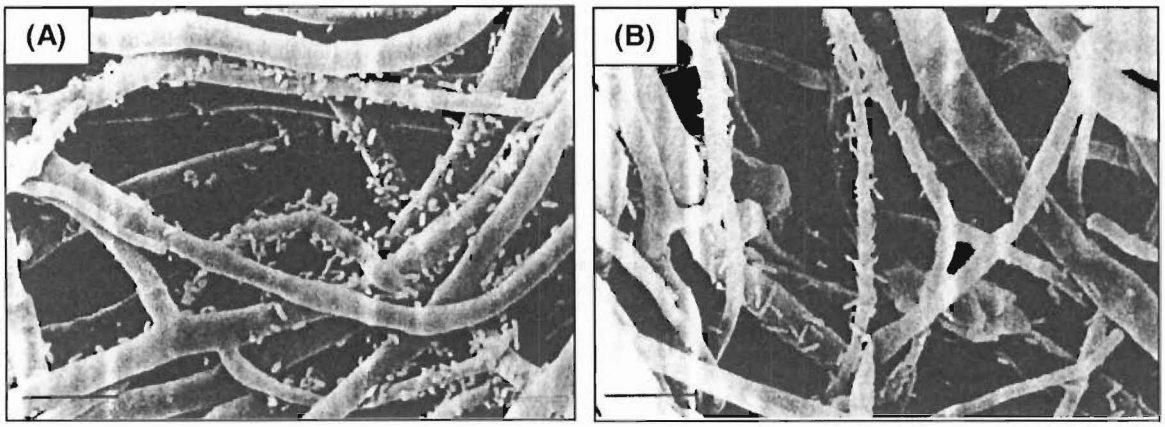

Figure 1-8 SEM micrographs of *A. bisporus* mycelia colonized by *P. putida* and *P. tolaasii* 30 min after application of the bacteria. (A) Wild type *P. putida* laying parallel to the hyphae and showing fibrillar material. (B) Wild type *P. tolaasii* attached perpendicular to the hyphae and showing also the fibrillar material (Rainey, 1991).

The surfaces of the bacteria in older natural infections appeared to be rougher or slightly 'corrugated' in SEM preparations and the reason for this is unknown. In the heavily infected parts of the tissues the bacteria appear to be in large 'clumps' held together by the rod-like connections (Preece and Wong, 1982b). These attached bacteria were observed more often on young hyphae than on old ones and the non-colonization of hyphal areas was associated with the age of the hyphae (Cochet, *et al.*, 1992). Possibly, the exuded metabolites chemotactically attract the bacteria towards the young hyphal surface where they multiply (Grewal and Rainey, 1991). The firm attachment of bacteria occurs almost immediately after these organisms are placed on the surface of the hyphae of *A. bisporus* (Preece and Wong, 1982b). These bacteria also caused the disappearance of the calcium oxalate crystals. The oxalate might be used under certain circumstances by these bacteria or by the fungus (Stanier, *et al.*, 1979). The attached bacteria (mostly *P. fluorescens*, *P. putida*) could be easily distinguished from *P. tolaasii* cells. *P. tolaasii* cells have a smooth surface and very clear flagella and the saprophytic bacteria exhibited a rougher surface (Masaphy, *et al.*, 1987). The smooth wild-type cells were observed attached and often orientated perpendicular to hyphal surfaces, in contrast to *P.*

putida, where the cells were orientated laying flat against the hyphae. The fibrillar rods were also frequently associated with the attached bacteria.

TEM studies of *P. tolaasii* infection

At the initial stage of infection, transmission electron microscopy (TEM) reveals *P. tolaasii* and *P. putida* were attached by a large amount of amorphous material associated with the bacteria, possibly polysaccharide slime. The amorphous material appeared to 'cement' bacteria firmly to the hyphal surface (Figure 1-9) and was found to be polysaccharides forming a continuum between the bacterium and the fungal surface (Rainey, 1991).

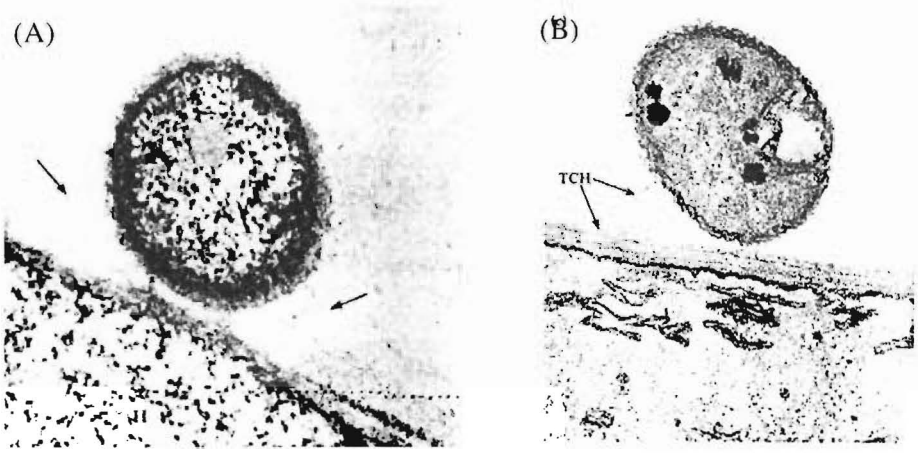

Figure 1-9 TEM micrographs of *A. bisporus* mycelium colonized by *P. putida* and *P. tolaasii*. (A) *P. putida* after 24 h, showing build-up of amorphous material between the bacterium and the hyphal surface. (B) the same as (A) but TCH stained to detect the polysaccharides indicating that the secreted material were polysaccharides. *P. tolaasii* provoked the same effect but the membrane appeared pulled away from hyphal wall, causing loss of cell contents because of the tolaasin production (Rainey, 1991).

At an early stage of infection by *P. tolaasii*, breakdown of the intercellular matrix and separation of the hyphae in the proximity of the invading bacteria occurs. This matrix erosion would indicate glucanase activity since this connecting matrix is an amorphous glucan that is partially continuous with the glucan of the outer hyphal wall layer. Disorganization of the organelles occurs and darkly stained material accumulates (Rahimian and Zarel, 1996). At more advance stages of infection, hyphae at the cap surface become filled with a homogeneous electron dense, darkly staining and granular material (indicating the presence of lipids and proteins bound by osmium oxides). Those beneath the main bacterial accumulation become disrupted and collapse forming a layer of lightly packed walls with only remnants of degenerate cytoplasm within. In these elements and in hyphae below the bacterial concentration, wall thickening is seen to occur and appear to form a barrier to exclude further pathogen attack of internal hyphae.

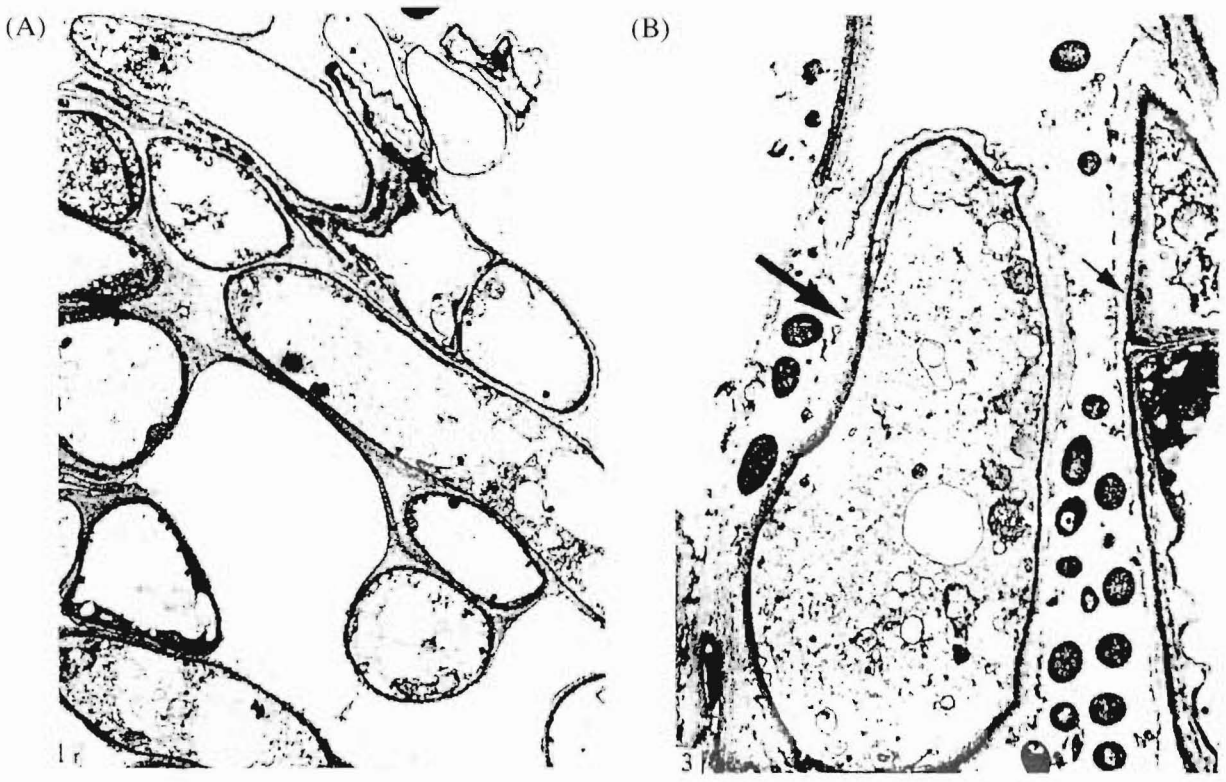

Figure 1-10 TEM micrographs of a transverse section of the cap of *A. bisporus* (Cole and Skellerup, 1986). (A) Healthy hyphae showing a large central vacuole, embedded in the connecting matrix with large intercellular spaces. X 4400. (B) *P. tolaasii* invasion results in breakdown of intercellular matrix and hyphal walls and disruption of the plasmalemma (large arrow). Surface hyphal cells are filled with amorphous darkly stained material (small arrow and under it). x 9900.

P. tolaasii was seen to invade mushroom cap hyphae to a significant extent being not only on the surface, but penetrating more deeply (Figure 1-10). Toxin production may be responsible for this intracellular damage observed. Since the bacteria and collapsed hyphae seen in deeper layers are clearly separated, it would indicate the mobility of such a toxin (Cole and Skellerup, 1986).

1.6 Management of bacterial blotch

As blotch is a worldwide problem, many management strategies have been devised. Presented below is a review of the current state of management bacterial blotch.

1.6.1 Resistance of *A. bisporus* strains

A wide diversity of *Agaricus bisporus* strains are commercialized that show differences in appearance, size, texture, and colour. However, many wild strains of *Agaricus* with different appearances can also be found in the forests. Their low yield or other complicated growth characteristics may make them less valuable for market purposes, yet these strains may hold promise for finding resistance to *P. tolaasii* and other diseases in the future (Fletcher, 1992).

The effect of the brown blotch disease has been studied, on a wide range of strains, (commercial hybrids and wild types) searching for resistant strains (Peng, 1986, Moquet, *et al.*, 1996).

A recent method to assess mushroom blotch resistance was developed by Olivier et al. (1997) and the derived studies indicate that cap resistance increases with flush number, regardless of the *A. bisporus* and *P. tolaasii* strain. Development of lesions did not depend on the morphological stage of the mushroom at inoculation and exhibited a range of disease reactions, ranging from highly resistant to highly susceptible.

1.6.2 Defense of mushrooms against bacteria

Many cultivated fungi have been shown to degrade living bacterial cells. Thorn and Tsuneda (1992) examined the interactions between various wood-decay fungi and living bacteria on agar and found that *Lentinus edodes* as well as 37 of 53 fungi tested had bacteriolytic ability. *L. edodes* was able to degrade *P. fluorescens* and *P. tolaasii* and examination by SEM showed a drastic degradation could be noticed at the vicinity of the young hyphae. Hyphal tips were the most active segments in the production of lytic enzymes (Tsuneda and Thorn, 1994).

The hyphal tips of *L. tigrinus* were most potent in bacteriolysis against *P. fluorescens*. Bacterial cells near the hyphal tips were disintegrated into small globules. *Pleurotus ostreatus* is also known to attack and degrade living bacterial cells on low- nutrient agar and on wood (Tsuneda and Thorn, 1994). The ultrastructural patterns of bacterial degradation by *Pl. ostreatus* differed between bacteria and *L. edodes* was apparent even between different strains of *P. tolaasii* (Tsuneda and Thorn, 1994). *A. bisporus* also produces extracellular bacteriolytic and fungolytic enzymes. It is capable of using heat-killed bacterial cells of *Bacillus subtilis* as its main nutrient source (Femor and Wood, 1981) and able to use other fungi and actinomycetes as a sole source of carbon, nitrogen and phosphorus (Fermor and Grant, 1985).

The decomposition of heat-killed *B. subtilis* cells by various filamentous fungi may take two forms: cytolysis, in which the bacterial cytoplasm was rapidly degraded, leaving apparently empty cell walls, and bacteriolysis in which the entire bacterial cell gradually disintegrated (Grant, *et al.*, 1986). Either specific carbohydrases or specific peptidases lysed cell walls of bacteria. The main bacteriolytic enzyme of *A. bisporus* active on *B. subtillis* was a p-N-acetylmuramidase (a carbohydrase) (Grant, *et al.*, 1984). No antimicrobial compounds have been described to be involved in the bacteriolysis of *P. tolaasii*. The Gram-negative cell envelope of *P. tolaasii*, differing from the *B. subtilis* envelope, may therefore be a problem for *A. bisporus* to use the same bactericidal enzymes.

Only a few indications of a possible defense mechanism are suggested by SEM illustrations where a homogeneous electron dense material appeared as a barrier excluding the pathogen from the internal hyphae (Preece and Wong, 1982b). Light micrographs of *A. bisporus* hyphae infected by *Verticillium* showed a layer of brown deposited pigments in the area close to the pathogen (Draght, *et al.*, 1996).

These brown pigments could be toxic compounds, such as melanins, produced by *A. bisporus* in order to inhibit growth of the pathogen (Hegnauer, *et al.*, 1985).

1.6.3 Blotch control strategies

Since *P. tolaasii* is generally introduced onto mushroom farms via the casing material and compost (Wong and Preece, 1980), sterilization of these substrates before its mixture with the spawn would appear to be a rational solution. However, the biological requirement for mycelial growth and basidiome initiation eliminates this possibility since mycelia cannot grow in sterile compost and sterilized casing does not stimulate basidiome production (Hayes, *et al.*, 1969).

Detection of *P. tolaasii*

A recent study has utilised PCR oligonucleotide primers for the detection of *P. tolaasii* targeting the genes required for tolaasin production (Lee, *et al.*, 2002). PCR in this study claims to distinguish *P. tolaasii* from other bacteria for studying ecology and monitoring potential contamination sites such as water, spawn, or other components involved in mushroom cultivation. Furthermore, phenotypic *in vitro* tests have been developed for the detection of *P. tolaasii* and these are presented in Chapter 2.6.

Control by regulation of environmental conditions

Environmental conditions play an essential role in the outbreak and severity of brown blotch disease and they can be manipulated to minimize the chance of an epidemic occurring. The relationship between relative humidity (RH) and temperature is the critical factor in disease control (Sinden, 1971) since this dictates whether or not the mushroom caps are wet. If the surface of the mushroom becomes wet, rapid multiplication of the bacterium is likely to result in visible disease symptoms within 12-24h (Nair and Bradley, 1980). Increases in disease severity resulting from changes in temperature and RH have frequently been reported (Gandy, 1967, Bashan and Okon, 1981) and the effect of fluctuating temperature is enhanced at the high RH levels maintained to prevent scaling of mushrooms. In theory, environmental control could be used to completely prevent blotch disease, but in practice this is not a realistic solution. In many commercial farms where *P. tolaasii* is endemic, the required level of precision in the control of temperature and RH cannot be achieved. Some authors also proved that temperature and RH changes were not able to avoid significantly the development of the disease (Preece and Wong, 1982a).

Apart from temperature and RH, other measures to minimize the disease have been reported. The ventilation of the cultivation room (Tu and Liao, 1989), treatment of the fruitbodies with aerated steam (Nair, 1974) combined with a special care in the preparation of the compost and casing (San Antonio, *et al.*, 1977) were proved to reduce the symptoms.

Chemical control

Control by addition of organic and inorganic compounds

Extensive research to find a suitable chemical for control of *P. tolaasii* has resulted in limited success. Historically, chlorine has been the most widely used and accepted chemical for controlling brown blotch disease (Royse and Wuest, 1980, Bashan and Okon, 1981), but there are problems associated with its use. The inherent instability of both sodium hypochlorite (Wong and Preece, 1985a) and chlorine dioxide (Wong and Preece, 1980) itself causes problems with storage and administration (Geels, *et al.*, 1991). In addition, the minimum concentration necessary for any notable effect on bacterial multiplication differs from one study to another and higher concentrations often cause browning of mushroom caps (Royse and Wuest, 1980), which is as unacceptable as the browning caused by the disease. Another study described the possibility of a reduction in the crop (Rutjens, 1977) and therefore the efficacy of this chemical is very limited.

Several other chemicals have been studied for their potential ability to control bacterial blotch, as sodium sulfite (Beelman, *et al.*, 1989), bipyridyl (Henry, *et al.*, 1991), sorbitol (Roy, *et al.*, 1995), formaline, glutaraldehyde, sodium metasulfite (Olivier, *et al.*, 1978, Lelley, 1984, Lelley, 1987, Vantomme, *et al.*, 1989), but none have found to be fully effective and non-toxic to mammals. Fumigation of the peat used to sterilize the casing layer with a non-explosive mixture of ethylene oxide has also been tried on a small scale and appears to be effective in the elimination of *P. tolaasii* (Nikandrow, *et al.*, 1982). However, this procedure also kills basidiome stimulating bacteria and is therefore not a practicable method for controlling brown blotch disease on commercial mushrooms farms. Furthermore, some disinfectants were tested such as bronopol (2-bromo-2-nitropropane-1,3-diol) (Wong and Preece, 1985b), N-cetylpyddinium chloride, benzalkonium chloride, cetrimide, panacide and chloramine T (Wong and Preece, 1985c). Bronopol was determined to be the most active compound against the pathogen because of its slow-acting bactericidal effect, destroying *P. tolaasii* in the mushroom casing. The inhibition was enhanced by the addition of Tween 80, EDTA and phenylethanol (Wong and Preece, 1985b).

Control by antibiotics

Although the use of antibiotics in mushroom growing has not yet been legally approved, some, such as kanamycin and streptomycin, reduced the bacterial count and did not affect the growth of mushroom mycelia (Munjal, *et al.*, 1989, Vantomme, *et al.*, 1989). Kasugamycin is another antibiotic produced by *Streptomyces kasugaensis* with bactericidal possibilities. It is effective in low concentrations and the toxicity towards mammals was low. The mode of action is the blocking of protein synthesis by hindrance of the coupling of amino acids to polypeptides on the ribosomes. It has been proved that kasugamycin is capable of significantly reducing the incidence and severity of bacterial blotch (Geels, 1995) and although kasugamycin has bactericidal and fungicidal properties, it seems not to reduce the mushroom yield.

Biocontrol

After less-than-total control with environmental and chemical control, a third option investigated is that of development of biological control methods.

Control by antagonist microorganisms

Control of the population size of *P. tolaasii* in the mushroom may be achieved by the addition to the casing of non-pathogenic microorganisms able to compete with *P. tolaasii*. Siderophore production to obtain the iron by competitors did not appear to be essential for successful biocontrol (Henry, *et al.*, 1991) so competition for the nutrients may play the most essential role in the antagonist mechanism. Potential control agents have been isolated from casing soils and mushroom farms suffering little or no blotch disease (Nair and Fahy, 1972b). Most of them were identified as belonging to the genus *Pseudomonas* (Fermor, *et al.*, 1991). Some strains of *P. fluorescens* (Healey and Harvey, 1989, Munjal, *et al.*, 1989, Khanna, *et al.*, 1990, Rainey, *et al.*, 1992), *Burkholderia cepacia*[1] (Nair, 1974), *P. putida* (Khanna, *et al.*, 1990), *P. reactans* (Grewal, 1991), *P. chlororaphis*, *P. fragi* were shown to reduce incidence of blotch in commercial trials (Nair and Fahy, 1972a). Other strains such as *Enterobacter aerogenes* (Nair and Fahy, 1972b), *Erwinia herbicola*, *Alcaligenes* sp. and *Bacillus subtilis* (Fermor and Lynch, 1988) were also shown to be active antagonists. The non-pathogenic form of *P. tolaasii* was tested as an antagonist of the pathogenic form with no effect against the disease development (Olivier, *et al.*, 1978).

Two different strains of *P. fluorescens*, which appear to compete successfully with *P. tolaasii* are currently being marketed under the name 'Conquer' in Australia and New Zealand (Rainey, *et al.*, 1992) and 'Victus' in the USA (Miller and Spear, 1995). Product reports by the marketing companies claim that Conquer and Victus act by competition with *P. tolaasii*. Regular, well-timed applications of Conquer in the bed area give an average 72% disease reduction, while Victus was also highly effective in increasing the total yield and mushroom quality.

It has been suggested that nematodes, e.g. *Caenorhabditis elegans*, may contribute to control of bacterial blotch by spreading antagonistic *P. reactans* which they use preferentially as a food source (Cayrol, 1975, Grewal, 1991) however, they can also can spread other bacteria that inhibit mycelial growth (*E. cloacae, B. cereus, E. amnigenus*) (Grewal and Hand, 1992, Grewal and Wright, 1992).

It is not surprising that a large proportion of the organisms tested for biocontrol ability against *P. tolaasii* are species of *Pseudomonas* since it is highly probable that, in the absence of specific antibiosis, the most effective control agents will be those which occupy a similar ecological niche to the pathogen.

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[1] Formerly described as *Pseudomonas cepacia.*

Biocontrol by bacteriophages

Another biological strategy tested to avoid increase in the number of *P. tolaasii* cells in mushroom beds was the addition of bacteriophages strongly aggressive to *P. tolaasii* and only moderately beneficial to *Pseudomonas*, which were added simultaneously for biocontrol (antagonist). A mixture of antagonistic bacteria and phages was sprayed on the casing and the first experiments showed that the effects of the antagonistic bacteria and of the phages were additive. The decrease in the symptoms was highly significant (>80%). However, several points have to be resolved before application on a larger scale can be envisaged. Despite the high practical significance of biological control, complete eradication of bacterial blotch is not realistic (Munsch, *et al.*, 1991). The efficiency of the bacteriophage to control the disease ranged, in eight independent experiments, from 30 to 80 %. Some strains of *P. tolaasii*, naturally resistant to the phage, were recently discovered (Munsch and Olivier, 1995). The possibility that *P. tolaasii* becomes resistant to the phages, the extreme precautions necessary for the manipulation of the virus suspension and the strict regulation by the law that forbid the use of the phages makes this strategy unmarketable as control for the disease.

New procedures for control

Selection and production of resistant strains

Crossing *A. bisporus* with other *Agaricus* spp. was not a viable method of producing improved or disease-resistant strains since the life cycle of the cultivated mushroom differs from that of related basidiomycetes. However, since the recent discovery of *A. bisporus* var. *burnettii*, a heterothallic and tetrasporic variety of the species (Callac, *et al.*, 1996), crosses have become possible. Some other advances have been made in the production of *A. bisporus* hybrids, but the production and selection of hybrids is a lengthy process and offers no immediate solution to the problem of disease control (Rainey, *et al.*, 1992).

P. tolaasii transformation

In some cases the most effective biological control agent has been shown to be a strain of the pathogen that has been genetically manipulated so that it is non pathogenic but otherwise isogenic with the disease-inducing organism (Rainey, *et al.*, 1992). At the present, a transformation system for *P. tolaasii* by electroporation has been successfully developed (Grewal, *et al.*, 1993). Tests with a toxin-minus, non-pathogenic strain of *P. tolaasii* have shown that such an organism may control low levels of pathogenic *P. tolaasii* (Olivier, *et al.*, 1978), but the relative effectiveness of the genetically manipulated strain against naturally occurring antagonists is still not tested.

1.7 Summary of current knowledge of blotch and blotch control

Blotch diseases are a worldwide problem with no truly effective control management strategies. Of the blotch diseases, the most research has been directed toward *P. tolaasii* and although *P. tolaasii* is not the only blotch causing bacterium, this knowledge and understanding may play an important role in helping elucidate how other blotch causing pseudomonads may cause blotch.

Because no effective management strategies exist, the possibility of finding an alternative method to chemical application has aroused interest in the furthering the understanding of epidemiology and pathogenicity determinants of bacterial blotch. Once this kind of knowledge is acquired, remedies to blotch control may present themselves. Presented in the remainder of this thesis is research aimed at gaining such an understanding.

1.8 Overview of objectives addressed in this thesis

To give a holistic framework to the research presented in this study, broad objectives have been outlined within two categories below. Please note that each chapter within these categories contains focused objectives pertaining to each section of research.

A. Determine the population structure of blotch causing organisms

A pilot study was proposed in which pseudomonads would be selectively isolated from a New Zealand national survey of all mushroom farms. This was proposed to fulfill two objectives: (i) obtain a representative sample of pseudomonad species causative of blotch diseases within New Zealand; and (ii) determine the relatedness of those pseudomonads able to induce blotch (termed blotch causing organisms (BCOs)) based on genetic and biochemical analyses.

B. Transposon mutagenesis analysis of blotch determinants

This section of study was divided into five sub-objectives: (i) to screen BCOs for pathogenicity determinants (identified in other bacterial studies) that may be involved in blotch; (ii) to develop successful methodologies for generation of transposon mutants of a selected BCO; (iii) to develop efficient screening processes for identifying mutants deficient in putative pathogenicity determinants; (iv) to identify Tn*5* mutants exhibiting a reduction in their ability to cause blotch discolourations in mushroom tissue bioassays; and (v) to characterise selected mutants by identifying transposon interrupted genetic regions within these mutants.

Chapter 2

CHARACTERISATION OF PSEUDOMONADS CAUSATIVE OF BLOTCH DISEASE WITHIN NEW ZEALAND

Two continuing observations initiated the pilot-study presented in this section of study: 1) New Zealand pseudomonads isolated from *A. bisporus* exhibiting 'ginger blotch' were variable in colony morphology and growth patterns; and 2) from an agronomist's point of view, 'ginger blotch' lesions exhibited variable discolouration, suggesting that either more than one organism was responsible for the discolouration or that the causative organism had variable virulence. Based on these observations, this study sought to address the molecular and phenotypic diversity amongst pseudomonads in the mushroom farm environment capable of causing 'ginger blotch' and other blotch related diseases of *A. bisporus*.

Chapter Outline

Presented within this chapter is research aimed at characterising pseudomonads causing blotch in New Zealand mushroom farms. An introduction follows in which the pseudomonads are reviewed in relation to their significance as a bacterial genus in mushroom cultivation. Following this is a review on blotch causing organisms with focus on the *P. tolaasii* physiological determinants of blotch disease, and the relatedness among mushroom associated pseudomonads described in previous studies.

INTRODUCTION AND LITERATURE REVIEW

2.2 The genus *Pseudomonas*

The genus *Pseudomonas* encompasses a ubiquitous and metabolically diverse group of Gram-negative, aerobic, chemoheterotrophic bacteria. Pseudomonads can be isolated from many natural habitats including sewage, soil, antiseptics, assorted foods, fresh water, marine environments and are also found in association with plants and animals. Due to this wide distribution of *Pseudomonas* in the environment and the ease by which these bacteria can be cultured, the genus today constitutes one of the best-studied bacterial grou*P*. The genus *Pseudomonas* can be divided into five groups on the basis of rRNA homology.

2.3 Fluorescent pseudomonads

The largest of the five groups, *Pseudomonas* rRNA group I (Palleroni, 1984), includes both fluorescent and non-fluorescent species. The fluorescent subgroup encompasses saprophytic species as well as plant and animal pathogens. Fluorescent pseudomonads are distinguishable by a somewhat variable but distinctive property, the production of a yellow-green, water soluble pigment, which diffuses into the medium and is fluorescent under ultraviolet light (Fava, *et al.*, 1993). The fluorescent pseudomonads were first distinguished into two biotypes based on their ability to liquefy gelatin and these two biotypes now bear the names *P. putida* (non-liquefying) and *P. fluorescens* (liquefying) (Stanier, *et al.*, 1966). Fluorescent pseudomonad species include *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. syringae*, *P. tolaasii* and *P. cichorii* and these have been extensively studied owing to their implication in areas such as bioremediation, biotransformation, biocontrol, and plant and animal disease. The majority of disease causing bacteria isolated from sporocarps and mushroom growing beds are fluorescent pseudomonads.

2.3.1 *Pseudomonas tolaasii*

The ability of *P. tolaasii* to cause blotch has been briefly described in Chapter 1, and here the physiology of the bacterium and its phylogenetic relationship amongst other pseudomonads is reviewed, *P. tolaasii* it is the most extensively reported pseudomonad associated with *A. bisporus* blotch diseases.

P. tolaasii microscopic characteristics

P. tolaasii is not unlike most other pseudomonads morphologically at the microscopic level in that the cells are Gram-negative, rod shaped and have polar flagella. However, *P. tolaasii* has been observed to exhibit microscopic morphological characteristics that are useful for taxonomical differentiation from other pseudomonads. This includes unique flagellar types whose composition differs from most of the rest of the *Pseudomonas*-flagella (Guilloritrondeau, *et al.*, 1996) and an outer membrane that has a composition that gives resistance to antibiotics such as Polymyxin B at 200 $\mu g.ml^{-1}$ that normally inhibits Gram-negative bacterial growth (Rainey, 1991).

P. tolaasii pathogenicity

P. tolaasii is able to colonise mushroom caps and induce the brown lesions on the cap surface (Figure 2-1).

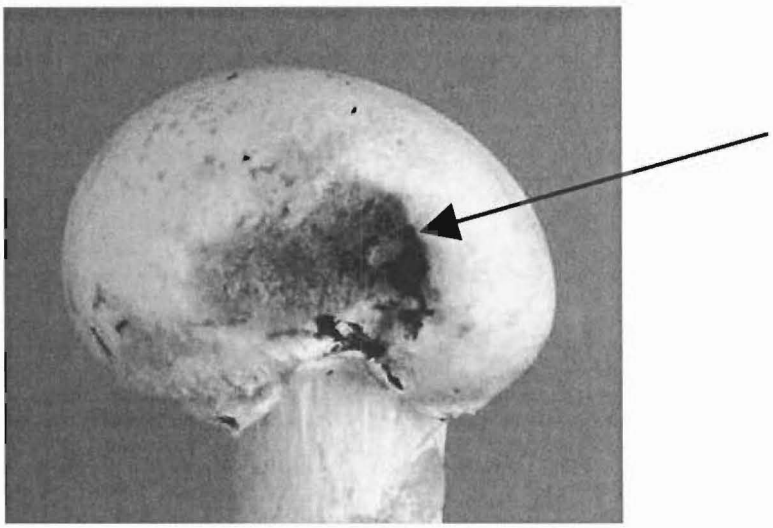

Figure 2-1 *P. tolaasii* brown blotch of *A. bisporus*. Dark pitted lesions are typical of *P. tolaasii* infection (arrow).

Tolaasin

The most prominently described of these extracellular compounds produced by *P. tolaasii* described is the lipodepsipeptide toxin, tolaasin. Tolaasin by itself can provoke pitting and browning in mushroom caps (Soler-Rivas, *et al.*, 1997b). Various modes of action have been described for tolaasin such as the production of cation selective channels in planar lipid bilayers and it having detergent-like properties that result in disruption of membranes and spreading of the disease[1].

[1] A full account of the physical properties and genetic and biochemical properties involved in tolaasin production is reviewed in the introduction of Chapter 3.

Other extracellular compounds produced by P. tolaasii

Recently, compounds other than tolaasin are being investigated because evidence suggests the existence of compounds that provoke symptoms similar to the brown blotch. These compounds are mostly extracellular enzymes such as proteinases or lipases, siderophores to capture the iron required for growth, exopolysaccharides to mediate cellular attachment to fungal mycelium and protect from desiccation, and toxins that disrupt hyphal membranes, thus providing a nutritional source from inside the cell[1].

2.3.2 *Pseudomonas gingeri*

In New Zealand, 'ginger blotch' disease symptoms (figure 2-2) are a more significant problem to mushroom cultivation than brown blotch commonly associated with *P. tolaasii* (NZ mushroom growers, pers. comm.). 'Ginger blotch' has been described in the literature as being caused by *P. gingeri*, and was first described as a new member of the *P. fluorescens* complex (Wong, *et al.*, 1982). Although *P. gingeri* demonstrates phenotypic similarity with *P. tolaasii* (e.g. during colony transition from pathogenic to non-pathogenic states (Cutri, *et al.*, 1984)), *P. gingeri* can be distinguished from *P. tolaasii* by the white-line-in-agar (WLA) assay and pitting assays (Wong and Preece, 1979) and the 2-ketogluconate and lipase tests (Wong, *et al.*, 1982). However, since its first description, *P. gingeri* has received limited attention as to its epidemiology and characterization as a species.

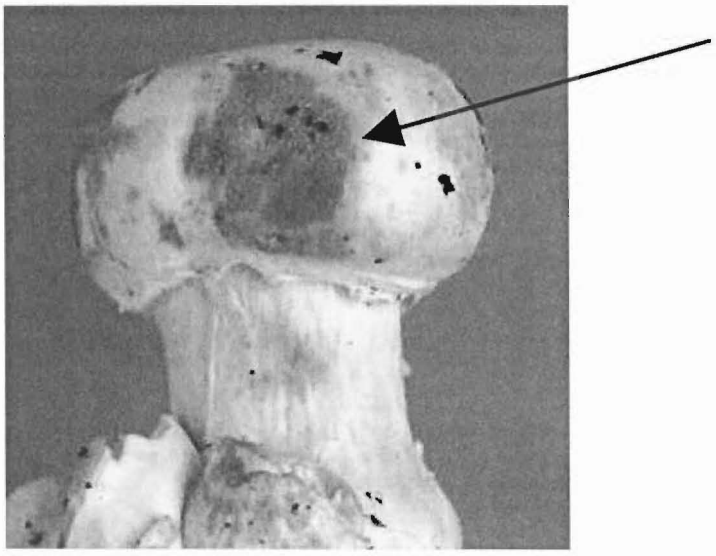

Figure 2-2 Typical 'ginger blotch' lesions (arrow) formed on *A. bisporus*. Mushroom sample from major New Zealand mushroom farm with lesions ginger in colour, but do not cause physical tissue damage to the cap surface.

[1] Again, such extracellular compounds are reviewed in Chapters 3 and 4.

2.3.3 Pseudomonas reactans

P. reactans is a strain that is considered to be closely related to *P. tolaasii* and *P. gingeri* because of similarity of environmental niche and biochemical phenotypic characteristics. *P. reactans* has been identified in pathogenic and non-pathogenic forms and the former has been described as being responsible for small, dark purple spots on harvested mushrooms (Wells, *et al.*, 1996). The non-pathogenic form, saprophytic on the mushrooms, has also been described as an antagonist of *P. tolaasii* preventing brown blotch formation (Grewal and Rainey, 1991, Soler-Rivas, *et al.*, 1999b), postulated to be via nutrient competition.

2.3.4 Pseudomonas agarici

P. agarici have been described as causing drippy gill and yellow blotch (Young, 1970, O'Riordain, 1972c, Gill, 1994) that is distinguishable from *P. fluorescens* (Unsworth and Preece, 1980) and *P. tolaasii* (Jin, *et al.*, 1994). Characteristics of isolated *P. agarici* strains indicated these to belong to *Pseudomonas* group III with a close resemblance to *P. cichorii* (Bateson, *et al.*, 1972). Because the prevalence of disease caused by *P. agarici* is low, extensive characterisation of continuing disease epidemiology and phylogenetic placement amongst the genus *Pseudomonas* is not fully resolved.

2.4 Colony transition between 'smooth' and 'rough'

Like most Gram-negative bacteria, pseudomonads possess the ability to undergo changes in their colony morphology. The transformation of pseudomonads from a 'smooth' colony form to a 'rough' colony form is well documented and may be due to an alteration in the composition of the cell lipopolysaccharide wall layers, although the mechanism by which this occurs is unknown (Govan, *et al.*, 1979). However, colony transformation is also often associated with changes in the genetic regulation of other phenotypic traits.

P. tolaasii colony transition

P. tolaasii colonies were originally described as fluorescent, white and mucoid on Kings medium 'B' (KB) and *Pseudomonas* agar F (PAF) media. However, it has been observed in many studies that *P. tolaasii* expresses a range of colony forms when cultured on solid media with the appearance of both smooth and rough forms (Zarkower, *et al.*, 1983, Cutri, *et al.*, 1984, Grewal and Rainey, 1991, Han, *et al.*, 1997, Wu, *et al.*, 1998). The wildtype colony is suggested to be the smooth phenotype with the rough variant arising spontaneously from sectors in the wild type colony after a few days of incubation (Grewal, *et al.*, 1995) (Figure 2-3). Smooth *P. tolaasii* colony forms, subcultured repeatedly on PAF, generally remained stable and non-fluorescent for 2-3 days, then became fluorescent, lost pathogenicity and exhibited rough colony forms (Cutri, *et al.*, 1984). The instability of smooth forms may explain why a loss of virulence in old cultures has been observed (Olivier, *et al.*,

1978). No difference in lipopolysaccharide banding pattern was observed between the pathogenic and non- pathogenic forms of *P. tolaasii* (Rainey, *et al.*, 1992).

The terminology smooth/rough implies only two forms, although by culturing the bacterium in a heterogeneous environment before growth on agar media, it is possible to observe an extensive range of intricately sculptured colony forms (Rainey, 1989). When smooth forms change to rough, the alteration in colony morphology is correlated with changes in biochemical characteristics. A gain in fluorescent pigmentation, inability to synthesize tolaasin or to hydrolyze casein, an increase in stability, a decrease in mucous production, and a faster growth rate than the smooth form has been observed (Grewal, *et al.*, 1995). Several hypotheses have been postulated to account for the instability of smooth forms and the supposed "irreversibility" that included common mechanisms for other bacteria such as spontaneous mutation, phase variation and loss of a plasmid (Cutri, *et al.*, 1984); however, plasmid loss was later shown to be not responsible for the switch (Rainey, 1989). Furthermore, a pathogenic form of *P. tolaasii* was obtained from the non-pathogenic form that was retrieved from prolonged storage at -70°C. This reversible transformation was later shown to occur at 1 in 10^6 cells (Grewal, *et al.*, 1995) indicating that colony transition is reversible.

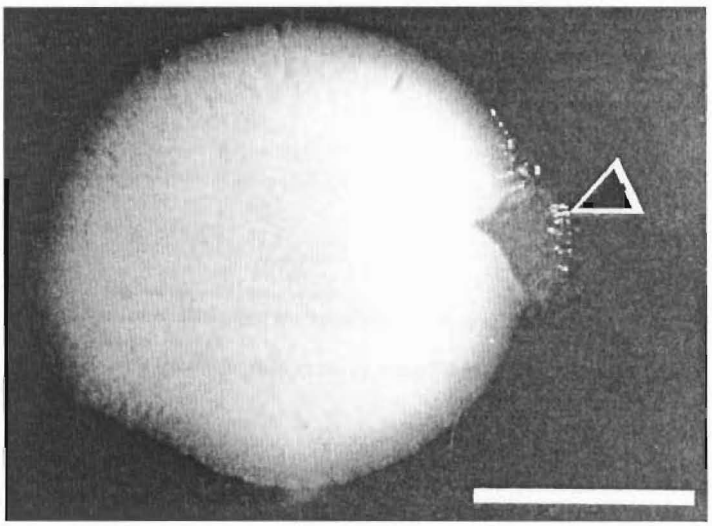

Figure 2-3 Growth of *P. tolaasii* on PAF medium after 7 days at 24°C, showing (arrow) a smooth colony phenotype emerging from the rough colony form on solid media. (Grewal, *et al.*, 1995).

P. tolaasii and *P. gingeri* colony transition

In the case of the mushroom pathogens *P. tolaasii* and *P. gingeri*, the transformation from rough to smooth is associated with many physiological and phenotypic changes, including loss of virulence (Cutri, *et al.*, 1984). Surprisingly the rough avirulent form of *P. tolaasii* has been shown to promote *Agaricus* mycelial growth in a similar manner to the saprophytic *P. putida* (Rainey, 1989). Stolp (1961) (in (Lelliott, *et al.*, 1966)), in considering the fluorescent pseudomonads as a homogeneous group, believed that the pathogenic forms might arise from the saprophytes via mutation. Regarding

this view as rather simplistic, Lelliott *et al.* (1966), suggested that the derivation of smooth virulent bacteria from the rough saprophytes may be driven by such selective pressures as limiting nutrient sources such as mushroom exudates. The rough forms appear to be more tolerant to environmental pressures such as nutrient stress, a situation common in casing where nutrient depletion occurs and a highly competitive environment exists (Rainey, 1989).

2.4.2 Genetic regulation of *P. tolaasii* pathogenicity determinants

PheN locus involvement in colony transformation

In an mutational investigation using Tn*5*km*lacZ* to understand the molecular mechanisms responsible for smooth-rough colony transition, a 3.4 kb genomic region in the smooth form of *P. tolaasii* was identified. This region was designated the *pheN* locus and was shown to complement the rough colony phenotype, causing reversion to the smooth colony phenotype (Grewal, *et al.*, 1995). Furthermore, it was shown that a functional copy of the *pheN* gene was required to maintain the smooth pathogenic phenotype, and conversely, the loss of the *pheN* gene (or its function) results in conversion to the rough phenotype.

The *pheN* locus (Figure 2-4) contains a 2727 bp open reading frame encoding for PheN, a 99 kDa protein (Han, *et al.*, 1997)). This protein shows similarity to members of a family of the two-component regulatory proteins[1] (Ronson, *et al.*, 1987, Frederick, *et al.*, 1997) which coordinately regulate the expression of multiple genes in response to environmental conditions. Sequence similarity of PheN to the two-component regulatory proteins extended to homology of the presence of histidine protein kinases (HPK) domains and their associated response regulators (RR).

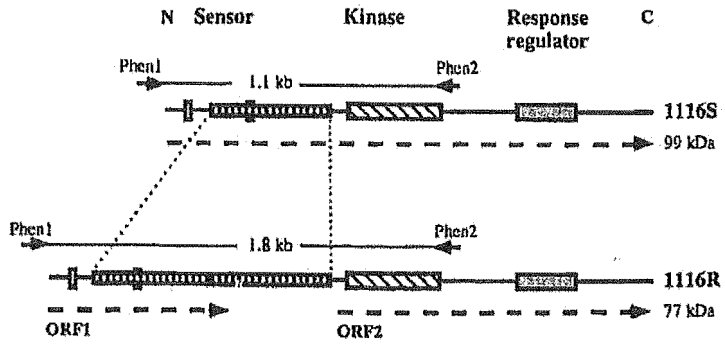

Figure 2-4 Diagrammatic representation of the DNA duplication event in the *pheN* locus. Small white squares are hydrophobic domains, hatched boxes are regions with homology to the conserved histidine protein kinase domain; stippled boxes are regions with homology to the conserved regulator domain; vertical striped boxes are 661 kb sequence (in a) normal size, present in the smooth form and in b) duplicated size, present in rough form). Translation products are indicated by horizontal dotted lines (Han, *et al.*, 1997).

[1] Two-component regulatory proteins are further discussed in Chapter 6.3.1 and 6.6

Recent studies showed that the phenotypic switch arises because of a 661 bp duplication within *pheN* (Han, *et al.*, 1997). This duplication event results in termination of the PheN ORF and the synthesis of a protein lacking 204 amino acids (77 kDa instead of 99 kDa) from the N- terminus of PheN. Since the N-terminal domain is the environmental sensor, loss of this domain is predicted to result in loss of PheN function. The mechanism of the DNA duplication event in *P. tolaasii* is still unknown (Figure 2-4) (Han, *et al.*, 1997),

2.4.3 Saprophycity and pathogenicity

Pathogen of mushrooms

P. tolaasii is best described as a pathogen of *A. bisporus*, although *P. tolaasii* is not host specific, as it can infect almost all species of mushrooms including: *Lentinula edodes* (Berk.) Pegler, or Shiitake (Tsuneda, *et al.*, 1995); *Pleurotus ostreatus* (Oyster mushroom or Hiratake) (Houdeau and Olivier, 1989); *P. eryngii* (Ferri, 1985); *Flammulina velutipes* (Enoki mushroom or Enokitake) (Ferri, 1985); and *Agaricus bitorquis* (Gandy, 1979, Upstone and Carter, 1979). In these *P. tolaasii* causes the same symptoms as on other mushrooms, including the inhibition of mycelial growth (Lee, *et al.*, 1996).

P. tolaasii has also been described as a saprophytic bacterium from the pear (Noval, *et al.*, 1993) and bean (*Phaseolus vulgaris*). The colonization of the bean roots by some *Pseudomonas* species such as *P. tolaasii*, *P. putida* and *P. aureofaciens* results in a selective induction of the synthesis of certain acid-soluble proteins involved in defense strategies (Zdor and Anderson, 1992). *P. tolaasii* can inhibit growth of plant pathogenic bacteria such as *P. syringae* pv. *coronafaciens*, *Clavibacter michiganensis* subsp. *michiganensis* and *Erwinia carotovora* subsp. *carotovora*, and the plant pathogenic fungus, *Fusarium solani* f.sp. *mori* (Shirata, *et al.*, 1995). However, *P. tolaasii* has also been found to be pathogenic toward plants such as the potato tuber (under laboratory conditions) (Shirata, *et al.*, 1995), *Nicotiana tabacum*, *Solanum* sp. (Rainey, *et al.*, 1991) and strawberry plants (Tanprasert and Reed, 1997).

2.5 Studies addressing relatedness of blotch causing organisms (BCOs)

Many previous studies addressing intra- and inter-species relationships of pseudomonads have included many of the blotch causing organisms. As a result of these various studies, many opinions have been formed as to the relationship of the pseudomonads associated with mushrooms. Presented in the following sections is a review of these various studies carried out and the results and conclusions drawn from the respective authors.

2.5.1 Early taxonomy of *P. tolaasii*

Assignment of *P. tolaasii* classification has always been under much debate with descriptions of assignment into various *Pseudomonas* biotype groupings (Stanier, *et al.*, 1966, Unsworth and Preece, 1980, Fahy, 1981, Zarkower, *et al.*, 1983). Later on, a new place as a separate species in the rRNA group V was suggested (Palleroni, 1986) but this place was disputed by other authors who reported that *P. tolaasii* rightly belongs to group I on the basis of rRNA / DNA homology (Rainey, *et al.*, 1992). However, *P. tolaasii*, like all bacterial speciation will continue to be unresolved until a unified species concept is defined (Maynard Smith, 1995).

2.5.2 Biochemical analyses of *P. tolaasii* and other BCOs

A detailed characterisation of *P. tolaasii* and other mushroom pathogens was performed using substrate utilization tests, electrophoresis of soluble proteins, and DNA:DNA hybridization experiments (Goor, *et al.*, 1986). Results showed that *P. tolaasii* formed a unique grouping, as did *P. gingeri*, saprophytic *P. fluorescens* biovar II, the so-called white line reacting organisms (including *P. reactans*), and *P. agarici* (Goor, *et al.*, 1986). In this same study, it was shown that *P. tolaasii* formed a grouping separate from both *P. fluorescens* and *P. reactans*.

Later, 2- and 3-hydroxy fatty acid profiling provided reproducible results for each of the six major groups of *Pseudomonas*, where *P. tolaasii* was determined to be included as group 1 (Stead, 1992). The same year, another study concluded from whole cell fatty acid analysis (FAA) the impossibility of classification in clusters because of finding a continuum of strains within the *P. fluorescens* complex (Janse, *et al.*, 1992). FAA is a technique that utilises the differences of fatty acid composition that occurs between different genus and/or species of bacteria and fungi. Fatty acids reside in the cytoplasmic membrane of gram-positive organisms and within both the cytoplasmic membrane and lipopolysaccharide (LPS) layer in the cell wall of gram-negative bacteria. Amphipathic lipids have a polar head and hydrophobic carbon chains that range between Cn_9 –Cn_{20} and these chains can be mono- or poly saturated and may contain chain branching, therefore, the potential for much diversity. Qualification of the different fatty acids are determined by extraction of FAs by saponification (addition of sodium or potassium hydroxide to form 'soap'), methylation to increase volatility and then the FAs are separated by Gas chromatography by partitioning the volatile molecules due to variances between mobile and stationary phase.

A later study using FAA and Biolog tests (Wells, *et al.*, 1996) provided data in which phylogenetic relationships were proposed in which *P. tolaasii* (pathotype A) was phenotypically identical to *P. fluorescens* biovar V but easily distinguished from other pathogens such as *P. gingeri* (pathotype B) and *P. reactans* (pathotype C) (Figure 2-5).

A different study (Kim, *et al.*, 1995) positioned *P. tolaasii* closer to *P. reactans* strains than (Wells, *et al.*, 1996) did. Other methods have also been used, including SDS-PAGE electrophoresis of whole-

cell proteins, results in a specific "fingerprint" for each species. Within the genus *Pseudomonas*, uniform and distinct patterns were found for some species, although in many species (including *P. tolaasii*) some strains exhibited significant protein electrophoretic heterogeneity (Vancanneyt, *et al.*, 1996a). Nowadays, some tests like Biolog and Biotype-100 systems are able to distinguish *P. tolaasii* from other *Pseudomonas* species (Grimont, *et al.*, 1996), but still it is not possible to have a proper taxonomic resolution of this extremely complicated genus.

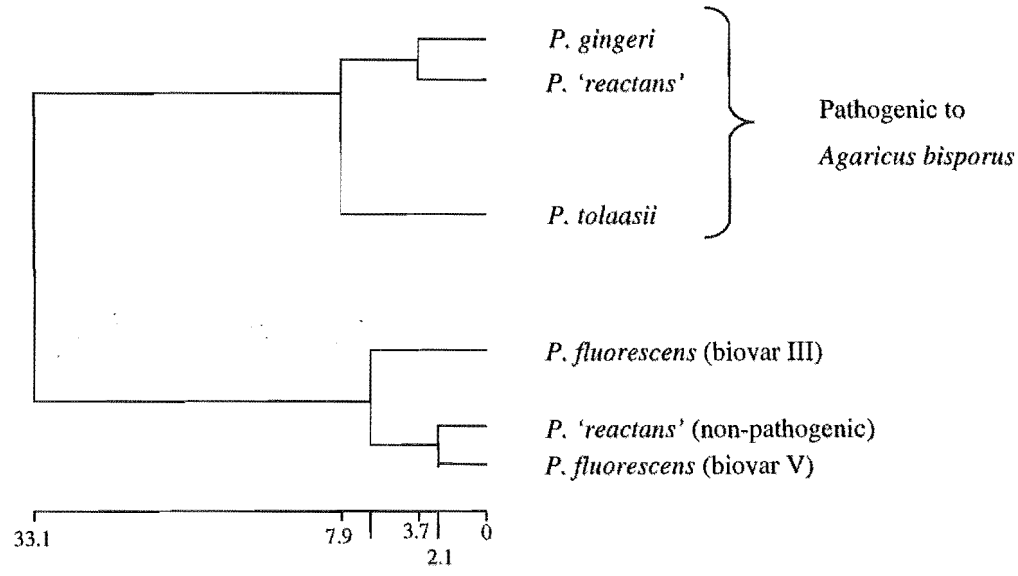

Figure 2-5a Dendrogram derived from cellular fatty acid profiles of some pathogenic and non-pathogenic strains of seven *Pseudomonas* groups (Wells, *et al.*, 1996).

2.5.3 Genetic studies that have included BCOs

16S rRNA analysis (Moore, *et al.*, 1996)

A consolidated effort compiling partial and nearly complete 16S rRNA sequences were analysed by several methods for a polyphasic study of the genus *Pseudomonas* (Moore, *et al.*, 1996). Twenty-one validated species of the genus were analysed, including multiple strains within each species. The first observation was that the natural intrageneric relationships among species inferred through sequence comparisons and cluster analysis did not show any obvious correlation with results derived from standard phenotypic criteria commonly used to group the species. Furthermore, this study outlined that the 16S rRNA gene from the genus *Pseudomonas* contains 1492 nucleotide positions, of which 148 positions are variable and 65 positions of these 148 reside within three hypervariable regions (Figure). These "*Pseudomonas* hypervariable (hv) regions" were defined as: hv 1. *E. coli* 16S rRNA gene sequence positions 71-95; hv 2. *E. coli* 16S rRNA gene sequence positions 455-475, and

hv 3. *E. coli* 16S gene sequence positions 998-1043 - which are located, respectively, within the rRNA regions V1: helix 6, V3: helix 18 and V6: helices P35-1 and P35-2 (Neefs, *et al.*, 1990). The "*Pseudomonas* hv 1 region" is recognised as being one of the most variable sequences in 16S rRNAs of bacteria across the phylogenetic spectrum (Woese, *et al.*, 1983, Gutell, *et al.*, 1985) and was deemed especially useful in inferred intrageneric lineages and discerning the 21 typed species of the genus *Pseudomonas* (Moore, *et al.*, 1996). Furthermore, this study showed that the cumulative aspect of 16S rRNA sequence databases is particularly important for the analysis of environmental isolates and the recognition of new sources of diversity as well as providing target sites for the development of 16S rRNA sequence-based strategies for the identification of species of the genus *Pseudomonas*.

Figure 2-5b schematic representation of *Pseudomonas* hypervariable regions found within 16S rRNA DNA defined as: hv 1. *E. coli* 16S rRNA gene sequence positions 71-95; hv 2. *E. coli* 16S rRNA gene sequence positions 455-475, and hv 3. *E. coli* 16S gene sequence positions 998-1043 (Moore, *et al.*, 1996).

Phylogenetic positioning of pseudomonads

The results of Moore *et al.* (1996) revealed that considerable intrageneric structure was discernible within the genus *Pseudomonas*, forming two distinct intrageneric divisions designated the "*P. aeruginosa* intrageneric cluster" and the "*P. fluorescens* intrageneric cluster" (Figure 2-6). This clear division has been supported in other studies using chemotaxanomic analyses with whole-cell fatty acid methyl ester (FAME) and phospholipid fatty acid profiling (Vancanneyt, *et al.*, 1996b). Of the BCOs, only *P. tolaasii* was investigated in this study and it was shown to cluster within the *P. fluorescens* lineage (as defined by Moore *et al.* (1996)).

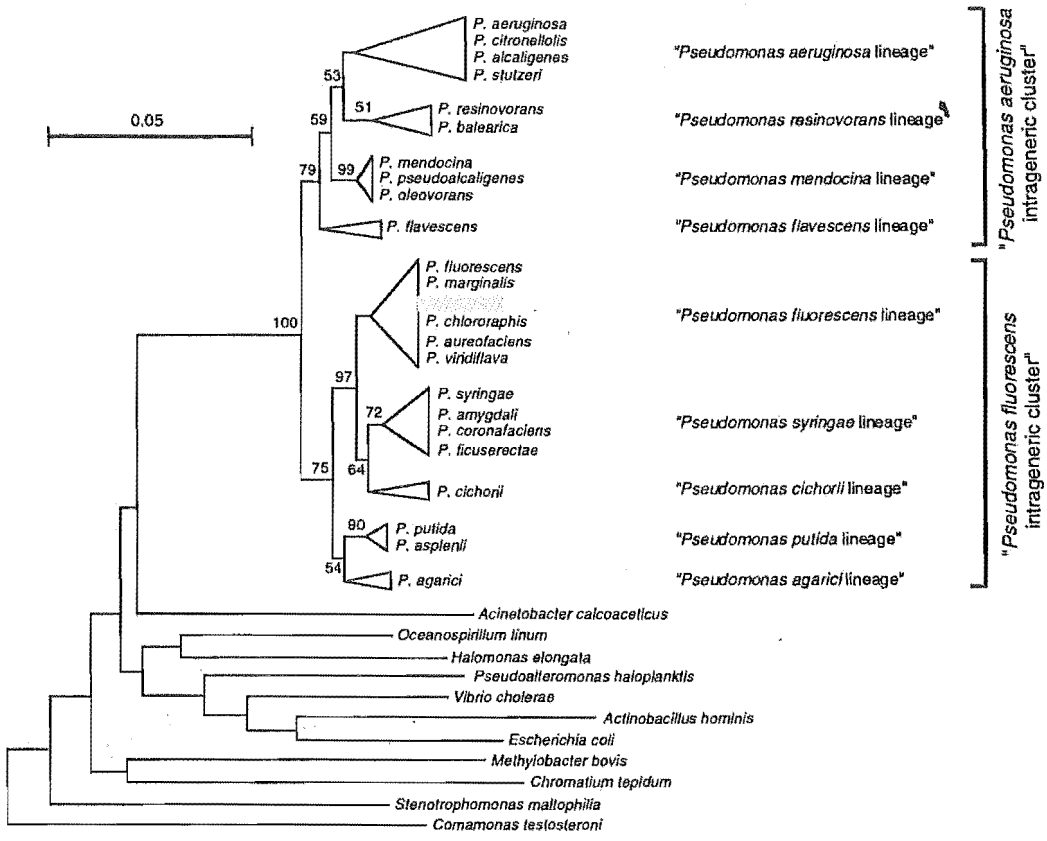

Figure 2-6 The inferred phylogenetic relationships among species of the genus *Pseudomonas* (sensu stricto). Evolutionary distances were derived from pairwise dissimilarities of the 16S rRNA gene sequences (Moore, *et al.*, 1996). Scale 0.05 is equivalent to 5% difference when distances from nodes added.

16S rRNA RFLP and REP-PCR analysis of *P. tolaasii*

P. tolaasii isolates were obtained from various mushroom species throughout Japan and phylogenetic and RFLP analyses based on partial 16S ribosomal DNA sequences indicated that *P. tolaasii* and *P. fluorescens* were close members of *Pseudomonas sensu stricto* (Thorn and Akihiko, 1996). Furthermore, this study showed all *P. tolaasii* isolates had identical RFLP patterns and partial 16S rRNA sequences and therefore it is suggested that *P. tolaasii* may represent a homogeneous grouping based on 16S rRNA. However, the technique of repetitive extragenic palindromic (REP) PCR (discussed below) showed variation amongst the *P. tolaasii* isolates investigated. The greatest variation were amongst *P. tolaasii* isolates from different mushroom crops (namely *Agaricus brunnescens*, *Flammulina velutipes*, *Lentinula* edodes, and *Pleurotus ostreatus*) and it was therefore postulated that the reason for so much disagreement in previous taxonomy of *P. tolaasii* published to date was a result of the variability in hosts from which *P. tolaasii* was isolated.

Repetitive extragenic palindromic (REP) PCR

During this study by Thorn *et al.* (1996), REP-PCR was used. REP elements are short intergenic repeated sequences that have been found in enteric bacteria, primarily *E. coli* and *Salmonella typhimurium* (Stern, *et al.*, 1984, Sharpies and Lloyd, 1990). REP sequences contain highly conserved central inverted repeats and can be divided into two classes that do not share significant homology. Class I consists of the repetitive extragenic palindromic (REP) elements (Stern, *et al.*, 1984) whereas Class II consists of the enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton, *et al.*, 1991). Comparison of the sequences of the conserved inverted repeats has allowed the derivation of REP and ERIC consensus sequences (Stern, *et al.*, 1984, Hulton, *et al.*, 1991) and in turn these sequences have been used for design of oligonucleotide primers (Versalovic, *et al.*, 1991). Using these primers it was shown that these sequences were present in a large variety of bacterial genera (Versalovic, *et al.*, 1991) and the PCR amplicons generate very characteristic patterns for different bacterial species when separated on agarose gels. Thus, REP and ERIC PCR have been used in many studies and constitute useful methods for fingerprinting bacterial genomes (De Bruijn, 1992). Thorn *et al.* (1996) showed that REP-PCR proved an effective technique for discrimination between similar *P. tolaasii* isolates identified by 16S rRNA nucleotide sequence and RFLP analysis. REP-PCR was efficiently applied to these blotch causing organisms and its applicability to deciphering closely related species was recommended.

Siderotyping for characterisation of *P. tolaasii* and *P. reactans*

Siderophore typing has been proposed as a rapid and efficient bacterial typing method for the discrimination of fluorescent pseudomonad strains (Meyer, *et al.*, 1997). Siderotyping is based on the recognition of the different types of pyoverdine (PVD), which is the typical fluorescent pigment and powerful siderophore of the fluorescent pseudomonads (Meyer and Hornsperger, 1978).

Siderotyping methods including pyoverdine isoelectric focusing analysis and pyoverdine-mediated iron uptake were used to analyse a collection of *P. tolaasii* and *P. reactans* isolates and revealed that *P. tolaasii* could be divided into two siderovars: the first siderovar showing homogeneity within *P. tolaasii* isolates from reference strains; and the second group comprising of Finnish *P. tolaasii* isolates identified by the white line test and *A. bisporus* pathogenicity (Munsch, *et al.*, 2000). This second grouping was shown to possess a pyoverdine that has been classified before as belonging to *P. aureofaciens* (Fernandez, *et al.*, 2001). The 40 *P. reactans* isolates also analysed in this study were shown to divide into eight siderovars, with some *P. reactans* isolates showing siderotyping similarity to previous *P. fluorescens* biovars II, III or V.

2.6 Experimental assays for studies and diagnosis of blotch disease

It is desirable to develop a methodology to efficiently screen isolates for the selected phenotype. Some experimental methods have been designed for identification of pathogenic pseudomonads causative of blotch in previous studies, although most have focused on *P. tolaasii*.

2.6.1 Pathogenicity test on entire mushrooms

This method was designed by Olivier *et al.* in 1978, whereby mushrooms were harvested and suspended on a 'wire net' with the stalk on wet cotton. The net with the mushrooms was placed inside a box and two drops (20 µl each) of a bacterial suspension were placed on the surface of a whole cap. Boxes were incubated at 16°C for 48 hours. This test has not only been used to compare pathogenicity of different strains, but also to study factors affecting the pathogenicity, the effect of chemicals and toxin extracts (Rainey, 1991). The effects of the bacterial suspensions or toxin extracts on the mushroom were scored visually according to the pitting and browning provoked.

2.6.2 Pathogenicity test on mushroom caps

Mushrooms were harvested, their stalk completely removed and the caps placed on a 'wire net' as for the experiments described above. A drop (20 µl) containing bacterial suspensions or tolaasin extracts were placed on the caps (Moquet, *et al.*, 1996). Caps were incubated and scored as described for the inoculation on entire mushrooms.

2.6.3 Mushroom tissue cube assays for *P. tolaasii* pitting

Gandy (1967) designed a method to compare the intensities of bacterial damage by *P tolaasii*. Fresh sporophores were harvested, their stipes detached and cap tissues cut in blocks of approx. 0.5 or 1cm^2 with the outer tissue peeled off. One drop of bacterial suspension was applied on the blocks and they were placed on glass microscope slides in a moistened covered plastic tray and incubated at 25°C. A conspicuous and rapid pitting of the mushroom blocks was observed by *P. tolaasii* within 10 min after a drop suspension was placed, long before any browning became visible. When the concentration of *P. tolaasii* inoculum was increased from 10 cells.µl^{-1} to 10^2 cells.µl^{-1} the pitting was easily visible in 5 min (Wong and Preece, 1979).

2.6.4 Mushroom cube discolouration assays for blotch causing organisms

Further to the above assay being effective for pitting of *P. tolaasii*, it has also been used for determination of *A. bisporus* discolouration by blotch causing organisms by lengthening the time of incubation, discolouration of tissue is observed between 24-48hrs (Figure 2-7).

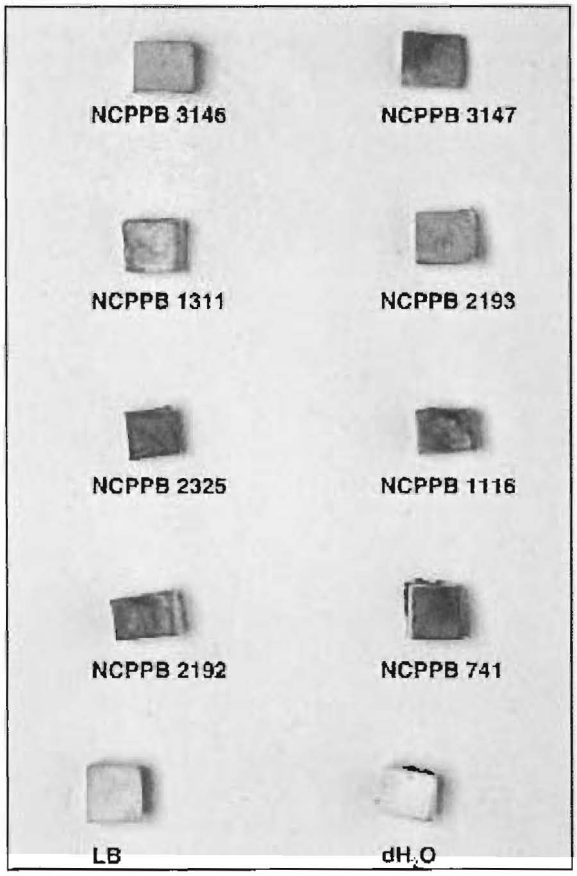

Figure 2-7 Mushroom block assays for the determination of *A. bisporus* tissue discolouration. Depicted are reference strains of: *P. gingeri* (NCPPB3146, NCPPB3147); *P. tolaasii* (NCPPB 2325, NCPPB1116, NCPPB2192, NCPPB 741, NCPPB 2193[1]); *P. reactans* NCPPB 1311; and negative controls on uninoculated LB and ddH_2O.

2.6.5 White line in agar (WLA) assay

The white line test has been described as a primary *in vitro* test for the identification of *P. tolaasii* (Rhodes, 1959, Wong and Preece, 1979, Zarkower, *et al.*, 1983, Palleroni, 1984, Janse, *et al.*, 1992). An interaction *in vitro* was observed between pathogenic forms of *P. tolaasii* and a saprophytic *Pseudomonas* species commonly found living on the mushroom cap (Wong and Preece, 1979). When these two types of bacteria are streaked several millimeters apart onto PAF and incubated at 25°C for 24-48 h, a white precipitate can be observed as a line between the colonies (Figure 2-8).

[1] *P. tolaasii* NCPPB 2193 is reported to be a 'non-pathogenic' *P. tolaasii* isolate **Goor** *et al.*, (1986).

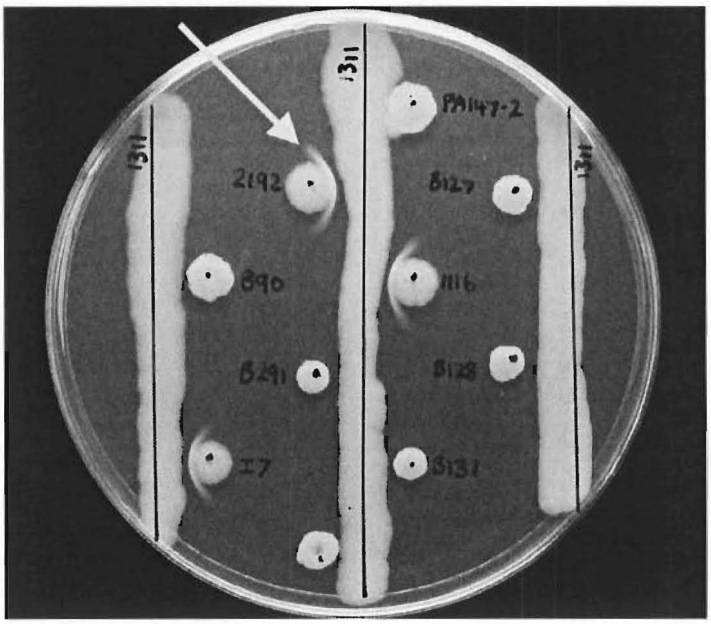

Figure 2-8 The white line in agar (WLA) assay showing the typical white precipitate that forms between *P. tolaasii* and white line inducing principle WLIP. Depicted type strains of *P. tolaasii* (2192, 1116) forming white precipitate (indicated by the arrow); whereas negative controls including *P. fluorescens* B90, *P. putida* B128 and *P. aureofaciens* PA147-2 do not form a WLA+ reaction.

The bacteria with which *P. tolaasii* interacts to produce the white line were designated as "*P. reactans*" (Wong and Preece, 1979). The white-line reaction is the result of a specific interaction between a diffusible compound produced by *P. reactans* (called the white line inducing principle, WLIP) and tolaasin. This reaction has been reproduced *in vitro* by use of partially purified and highly purified components of tolaasin (Rainey, *et al.*, 1992). Neither the mechanism whereby the white line is formed nor the biological significance of the reaction is fully understood, however WLIP like tolaasin, is a lipodepsipeptide (Mortishire-Smith, *et al.*, 1991). The structure of WLIP was almost identical to viscosin (compound synthesised by *P. viscosa* (Neu, *et al.*, 1990)) in every respect except for LeuS, which has the D configuration in WLIP and the L configuration in viscosin (Mortishire-Smith, *et al.*, 1991).

RATIONAL OF THIS SECTION OF STUDY

New Zealand observations of ginger blotch disease

In 1995 and 1996, New Zealand mushroom farms had a severe disease epidemic that caused millions of dollars worth of damage due to mushroom spoilage and consumer rejection of discoloured mushrooms in the market. The disease was identified as 'ginger blotch'. However, this diagnosis was based on the observation that the disease was undoubtedly caused by bacteria, but this bacteria was not *P. tolaasii*; based on phenotypic identification using the WLA and mushroom cube bioassay. Therefore, the bacteria was assumed to be *'P. gingeri'* because the disease lesions were concurrent with those described in previous literature (Wong, *et al.*, 1982), that is, the lesions were ginger in colour and no obvious tissue damage was observed. However, the causative organism(s) was never identified and the disease was treated by controlling environmental conditions for general bacterial blotches [1]. Since 1996, there has not been a repeat of this epidemic, although 'ginger blotch' is often present and remains a consistent problem to mushroom growers and identification of causal agent(s) needed addressing. Therefore, an aim of this thesis was to gain insight into the population dynamics of the causal organism of 'ginger blotch' throughout New Zealand.

Prior understanding on P. gingeri *homogeneity*

The disease symptom of 'ginger blotch' has been reported within literature as being caused by the pseudomonad, *P. gingeri* (Wong, *et al.*, 1982). *P. gingeri* isolates have been investigated and described as a new member of the *P. fluorescens* complex (Wong, *et al.*, 1982) and shown to have some phenotypic similarity with *P. tolaasii* in respect to colony transition from pathogenic to non-pathogenic states (Cutri, *et al.*, 1984). Apart from these descriptions, further studies have reported only on phenotypic discrimination of *P. gingeri* from *P. tolaasii* (Wong, *et al.*, 1982) and other similarly related pseudomonads (Goor, *et al.*, 1986, Wells, *et al.*, 1996). However, since its first description, *P. gingeri* has received limited attention as to its epidemiology and characterization as a homogeneous species. Personal communication with mushroom growers in both New Zealand and Australia has established that that 'ginger discolourations' far exceed the observations of classical 'brown blotch' with pitting, caused by *P. tolaasii*. Unfortunately, such 'on farm' observations are not published often enough and, a discrepancy exists between what the literature reports as the dominant bacterial disease, and what is actually observed amongst mushroom growers. To the knowledge of this author, no prior research has been carried out to determine if 'ginger blotch' lesions are consistently caused by the organism described as *P. gingeri*.

[1] Manipulation of environmental conditions for blotch control is described in Chapter 1.6.3

2.7 Objectives for Chapter 2

Therefore, based on two continuing observations: 1) pseudomonads isolated from so-called 'ginger blotch' *A. bisporus* lesions were variable in colony morphology and 2) growth patterns; and blotch lesions exhibited variable discolouration, suggesting that more than one organism was responsible for the discolouration (or that the organism had variable virulence), this first section of study was aimed at elucidating the population dynamics of pathogenic pseudomonads on New Zealand mushroom farms with an underlying agenda of determining the homogeneity of the organism responsible for 'ginger blotch'.

The hypothesis to be tested was '*a number of different Pseudomonas species possess the ability to induce blotch discolourations of* A. bisporus *mushroom tissue*'; and was tested within the following objectives:

1 To select mushroom farms representative of topographical distribution throughout New Zealand and selectively isolate pseudomonads from mushroom farms exhibiting 'ginger blotch' disease symptoms.

2 Design *in vitro* mushroom cube tissue bioassays and use these to determine the ability of the isolated pseudomonads to cause blotch discolourations of *A. bisporus* tissue.

3 Select pseudomonad isolates from a milk factory environment and determine if bacteria from a different environmental niche have the potential to cause blotch discolourations.

4 Select a representative sample of pseudomonads from objective 2 & 3 that: a) induce variable blotch discolourations; and b) have distinguishing biochemical and genetic profiles

5 To carry out comparative studies to attempt to assign species similarity and phylogenetic relationships of isolates selected in objective 2 & 3.

6 Analyse biochemical and genetic information and assess commonalities among the pseudomonads to propose why they are able to cause blotch.

METHODS & RESULTS

2.8 Selective isolation of pseudomonads from NZ mushroom farms

Pseudomonads were selectively isolated from blotched mushrooms and various substrates from a major mushroom farm and three minor mushroom farms within New Zealand (Figure 2-9). Farm selection targeted farms that were willing participants in sending samples for analysis and had been experiencing blotch symptoms. Sampling from mushroom farms was carried out during December 1999.

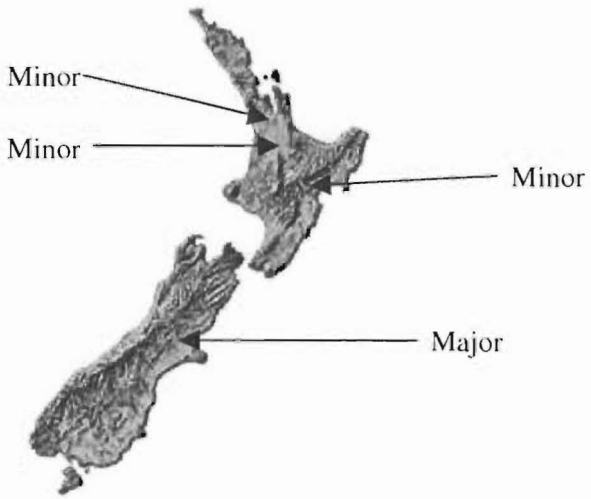

Figure 2-9 Geographical locations of the one major and three minor mushroom farms in NZ where pseudomonad isolates were obtained.

Sampling locations within farms

Samples were taken from either mushroom tissue exhibiting blotch, putative inoculation points (compost, casing, and materials within) and water samples from irrigation systems used to inoculate the beds. A section of tissue was excised from mushroom caps exhibiting blotch discolourations and was placed into a McCartney tube containing sterile KB medium (10 ml). One gram of compost or casing material was placed into a McCartney tube containing sterile KB medium (10 ml). Water samples from water reservoirs and frequently used taps on mushroom farms were collected in sterile McCartney bottles. Samples were maintained on ice upon collection and during transport to the laboratory, a period not exceeding 2 hr. Samples were incubated for 24 hr at 28°C before an aliquot was applied to Gould's agar medium (Gould, *et al.*, 1985). Gould's agar was chosen as it contains the

Pseudomonas selective agents trimethoprim and sodium lauroyl sarcosine. Trimethoprim is selective as it inhibits DNA synthesis and is active against many Gram-positive cocci and most Gram-negative bacilli (Yao and Moellering, 1999) and sodium lauroyl sarcosine prevents the growth of Gram-positive bacteria (Gould, *et al.*, 1985). Furthermore, sucrose and glycerol are included to create an osmotic stress that selects for *Pseudomonas*. Despite this selective environment, other colony morphologies were observed, although these were not selected for further purification.

Colony phenotypes that resembled *Pseudomonas* species[1] were selected and individual purification by passage onto fresh Gould's medium. Considering that colony transition (and coherently pathogenicity) occurs after prolonged *in vitro* incubation on agar plates (Cutri, *et al.*, 1984), isolates were cultured within 24hrs and sub-cultured, purified and cryogenically stored within 72 hrs of isolation. From the isolates exhibiting API 20 NE profiles belonging to the genus *Pseudomonas*, 50 purified isolates were selected from the major mushroom farm, and 15 isolates each from each of the three minor mushroom farms, giving a total of 95 isolates from around New Zealand.

2.9 Development of a repeatable mushroom tissue bioassay

A mushroom cube bioassay was developed for use in this study based on the technique devised by Gandy *et al.* (1967) and only 'healthy' first flush *A. bisporus* mushrooms were used. Healthy in this context is defined as first flush sporophores, devoid of any discolouration and harvested only from sheds exhibiting no disease symptoms. Mushroom cube bioassays were set up as depicted in Figure 2-10.

[1] *Pseudomonas* isolates of *P. tolaasii* 2192, 1116, 2325, *P. gingeri* 3147, 3146, *P. reactans* 1311, *P. fluorescens* SBW25, B90, *P. aureofaciens* PA147-2 were grown for comparative analysis of colony selection.

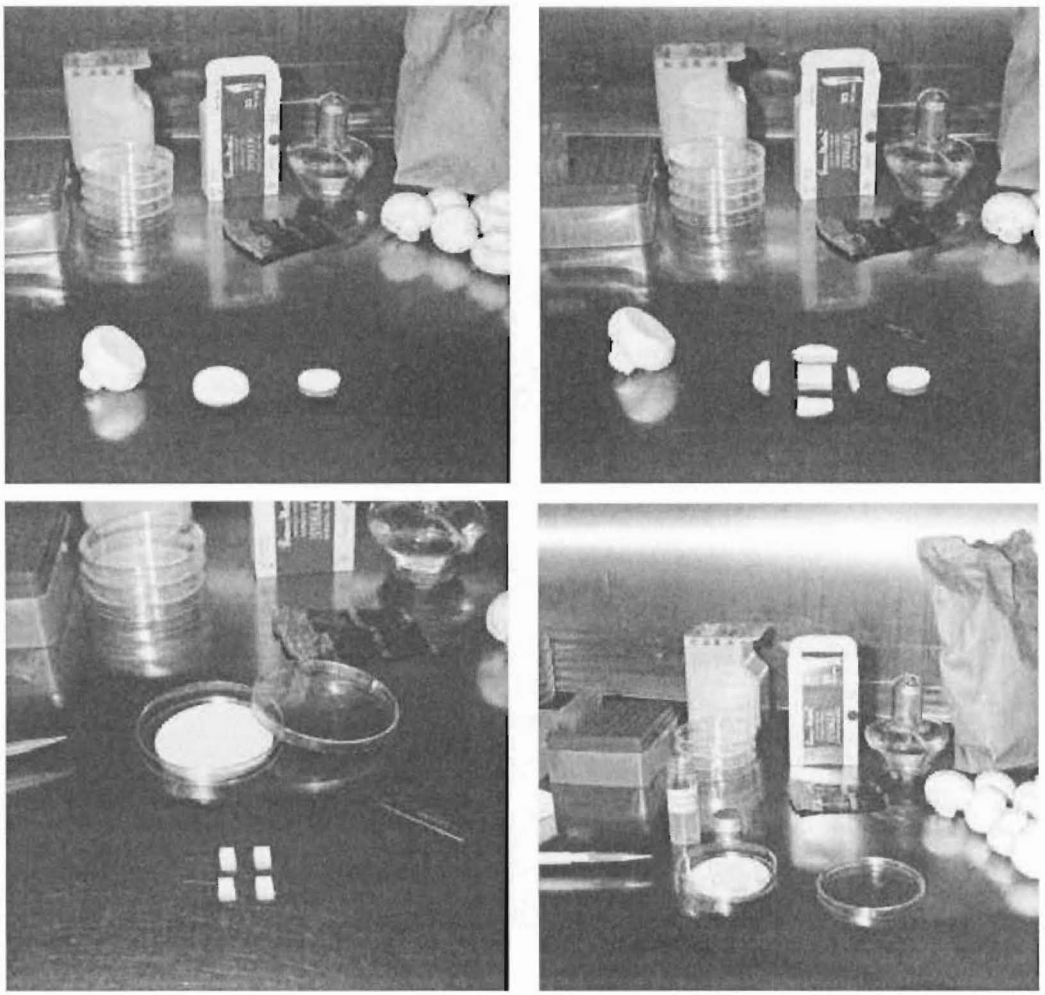

Figure 2-10 The four stages of preparation of the mushroom tissue bioassay. A) The outer skin of the mushroom was removed using a sterile scalpel. B) A central portion of the mushroom tissue was excised and C) cut into four cubes. D) These cubes (1 cm^3) were placed into a sterile 75mm Petri dish containing a 50 mm paper filter dampened with 800 µl of sterile ddH_2O. The four cubes were placed 2 cm apart in each Petri dish.

Assessment of blotch using mushroom cube bioassays

All 95 bacterial isolates were tested to determine their ability to induce discolouration of *A. bisporus* tissue in the optimised mushroom cube bioassay procedure outlined below. Furthermore, pseudomonads isolated from a milk processing plant (Reid, 1997) were also included to determine whether pseudomonads from a different niche could cause blotch discolourations of *A. bisporus*.

Determining cfu.ml^{-1} of inoculum

Bacterial strains were cultured in Luria Bertani (LB) medium (Appendix II) at 28°C for 16 hrs. Following these incubation conditions, cells were harvested, resuspended in ddH_2O and based on calculation of OD_{600} readings; cultures were diluted with ddH_2O until all cultures exhibited an

identical OD_{600} reading that equated to a cell-density of ca. 1 x 10^9 cfu.ml^{-1}. This cell-density was confirmed by serially diluting an aliquot from each isolate and performing plate counts to calculate corresponding cfu.ml^{-1}. In any given determination of cell-density, it is difficult to ensure absolute cfu.ml^{-1} thus the results often varied between 1x10^7 to 1x10^8 cfu.ml^{-1}, but were considered sufficient for the purpose of initial screening of the 95 isolates.

Inoculation of mushroom cube bioassays

From the above cultures (1x 10^9 cfu.ml^{-1}), 30µl was suspended in 120 µl of ddH_2O and a 50µl aliquot was placed onto each three of the four cubes (Figure 2-101). This 50µl aliquot provided of a cell-density of ca. 1 x 10^7 cfu.ml^{-1} or an individual cube inoculation of ca. 1 x 10^5 cfu. The fourth cube was inoculated with a 50 µl control of uninoculated ddH_2O. Petri dishes were sealed with parafilm and incubated at 20°C for 48 hr and results could be visualised at defined time intervals; generally 12, 24, and 48hrs (Figure 2-11).

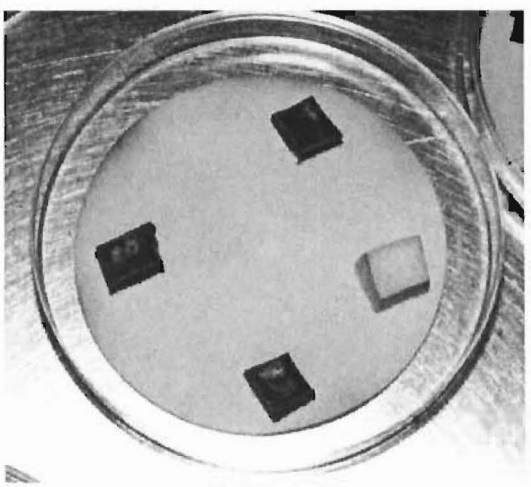

Figure 2-11 Example of blotch discolouration by NZ103 showing three consistent blotch discolourations and the negative control cube inoculated with ddH_2O.

Motility of BCOs between mushroom cubes in bioassay

Motility was investigated for selected BCOs by removing a cube exhibiting blotch from a previous assay and placing within the above defined mushroom cube bioassay conditions in close proximity of four other mushroom cubes (Figure 2-12). After 48hrs, blotch discolouration was assessed and was shown that a graduated decline in discolouration occurred the further away from the original mushroom cube (Figure 2-12). The graduated blotch discolouration was considered a result of motility from the originally inoculated mushroom as NZ103 has not been identified to have diffusible pathogenicity determinants defined within this thesis. The decrease in blotch discolouration was deemed a result of fewer colonizing bacterial numbers colonizing cubes further away from the primary cube; however, it should be noted that bacterial numbers were not determined.

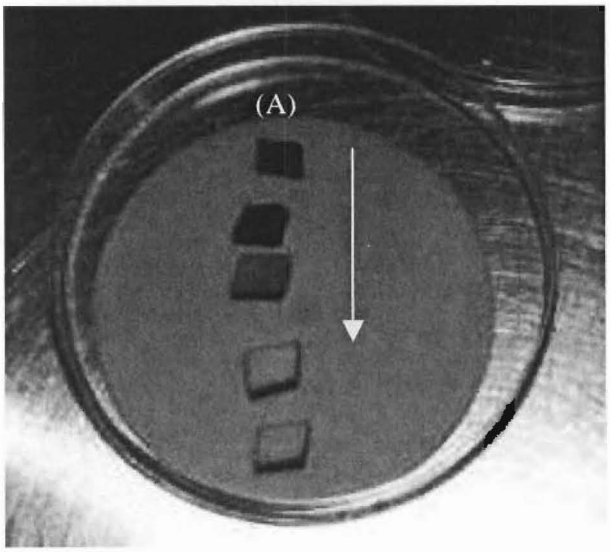

Figure 2-12 BCO motility between mushroom tissue cubes by BCO (NZ103). The primary cube (A) was inoculated only, and after 48 hrs, observation of blotch discolouration of uninoculated cubes becomes visible in a gradient of blotch discolouration.

Therefore, mushroom cube bioassays were set up with the four mushroom cubes arranged 2 cm apart on moistened filter paper. The distance between the cubes was an attempt to reduce the amount of cross-contamination by motile pseudomonads. After 48h the uninoculated cubes were assessed and generally showed slight discolouration in comparison to the originally inoculated cube, however, the further the distance from the infected cube, the less the discolouration.

Assessing degree of bioassay discolouration

Following incubation, mushroom caps inoculated with bacterial cultures were scored for the degree of blotch discolouration on a scale of B1 to B9 (where B = blotch) (Figure 2-713). To ensure comparable and internationally repeatable results, a color scale was developed using the revised Munsell standard soil colour charts (issued 1957) where: B1 = Hue 2.5Y 8/1; B2 = Hue 2.5Y 7/2; B3 = Hue 2.5Y 6/3; B4 = Hue 2.5Y 5/4; B5 = Hue 2.5Y 4/6; B6 = Hue 2.5Y 4/4; B7 = Hue 2.5Y 3/3; B8 = 2.5Y 3/2; and B9 = Hue 2.5Y 3/1).

Because *A. bisporus* tissue has a high propensity for variation in the degree of discolouration between mushrooms from different sources (Jolivet, *et al.*, 1995, Leeuwen and Wichers, 1999), all mushroom cube bioassays of bacterial isolates were repeated in triplicate using different sources of *A. bisporus* tissue from different sheds and different mushroom farms. That is, mushrooms were hand picked form beds of first flush sheds exhibiting no disease symptoms. Mushrooms were chosen from different areas around the farm, and within sheds in order to provide a random sampling. When bioassays were set up, mushrooms from different sources were sliced into cubes (figure 2-10) and one of the four cubes were placed into separate petrie dishes; thus each bioassay was performed on three

different sources of mushroom. Only results that showed consistent discoloration between these three cubes were recorded. Three replicates of tissue discoloration were performed for each isolate and results generally did not vary more than one blotch scale (ie B2-B3). This labour intensive procedure was performed in an attempt to control for the pathogenicity of the bacterial isolate, not the ability of *A. bisporus* discolouration. Furthermore, tissue degradation, coloring of filter paper and discolouration of the control cube were recorded after 48 hr.

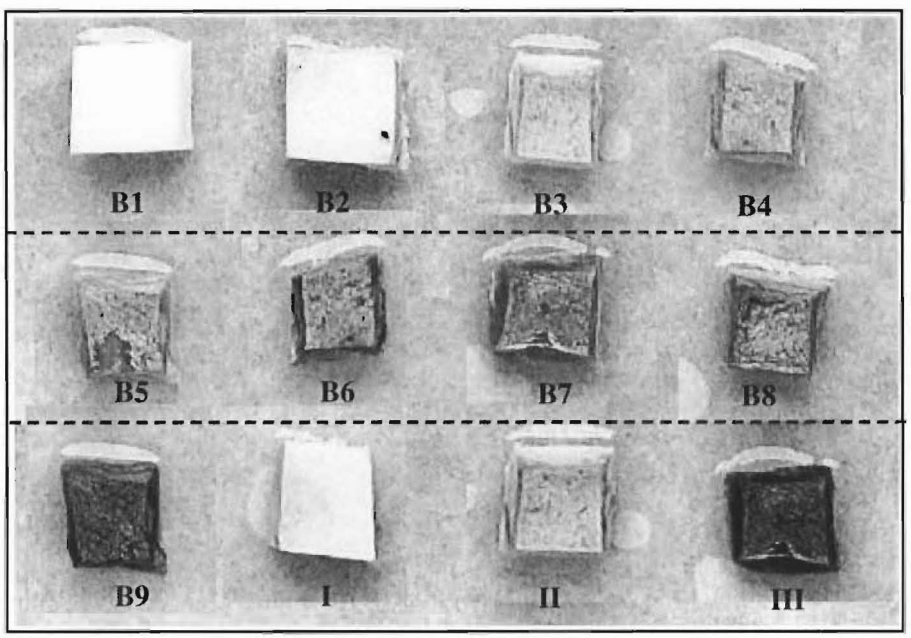

Figure 2-13 Selected cube pathogenicity bioassay results from BCO isolates capable of inducing discolouration of *A. bisporus tissue*. Pictured are cubes within the assigned colour scale B1-B9 (B1 = cube inoculated with LB alone). Reference strains are included for comparison: I, *P. reactans* NCPPB 1311 (B2); II, *P. gingeri* NCPPB 3147T (B5); III, *P. tolaasii* NCPPB 2192T (B9).

Results of the tissue bioassay showed that 76 (80%) of the 95 bacterial isolates caused discolourations of *A. bisporus* tissue to varying degrees (B2, 3.1% (n=3); B3, 36.8% (n=35); B4, 10.5% (n=10); B5, 11.6% (n=11); B6, 11.6% (n=11); B8, 2.1% (n=2); B9, 4.2% (n=4)). Pseudomonads capable of causing discolouration in bioassays were termed blotch-causing organisms (BCO) for future discussion purposes. The remaining 19 of the 95 isolates exhibited discolourations consistent with negative controls (B1) and were therefore, considered as non-pathogenic toward *A. bisporus*.

2.10 Refined selection of 33 isolates for further study

As evident from the results above, it is clear that many different blotch discolourations are present, not just the typically defined brown and ginger blotches described by other studies. Therefore, based on these results a new aim of this chapter was to characterise a number of these pseudomonads able to

cause variable blotch discolourations. As it was not realistic to fully characterise 95 bacterial isolates, it was decided to refine the selection to a smaller number for a focused study. Pseudomonads were selected for further study based on the following criteria: 1) they were representative of a variable discolouration of *A. bisporus* tissue in bioassay; and 2) they exhibited variance in colony morphology and growth patterns; initially 45 isolates where chosen. Repetitive extragenic polymorphic (REP) PCR (De Bruijn, 1992), was performed on all isolates to determine clonality[1], revealed that only 33 of the 45 selected BCOs were unique when compared to each other (Figure 2-14). Three milk-industry pseudomonads that could also induce blotch discolourations were part of these 33 REP-PCR discriminated pseudomonad isolates and were included in the following investigations.

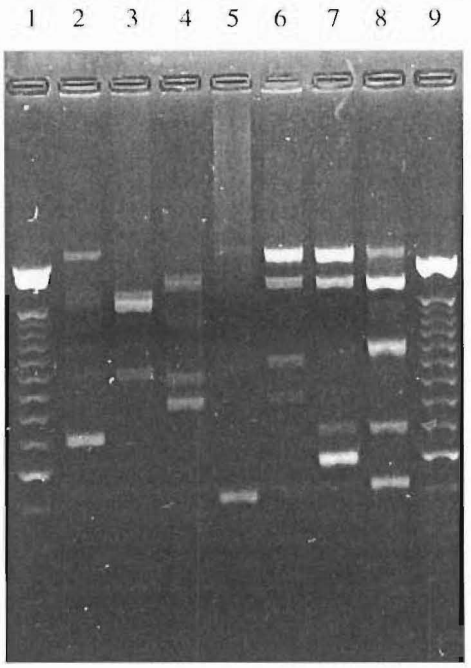

Figure 2-14 REP-PCR banding profiles demonstrating differences between selected BCO isolates. Lanes 1 and 9 = 100 bp ladder (LIFE TECHNOLOGIES); Lane 2 = NZ 101; Lane 3 = NZ 097; Lane 4 = NZ 102; Lane 5 = NZ 024; Lane 6 = NZ 060; Lane 7 = NZ 062; and Lane 8 = NZ 052.

2.11 Lipodepsipeptide (LDP) production

To determine if the 33 REP-PCR defined strains produced tolaasin or white-line-inducing principle (WLIP), lipodepsipeptide (LDP) production was assessed using the white-line-in-agar (WLA) assay (Wong and Preece, 1979). Bacterial colonies were toothpick-inoculated at a distance of approximately 7 mm from a streak of the indicator bacteria, either *P. reactans* NCPPB 1311 or *P. tolaasii* NCPPB 2192T on *Pseudomonas* Agar F (PAF) (Appendix II). After incubation at 28°C for a period of 24-48

[1] Discussed in Sections 2.5.3, and Appendix I(iv)d

hrs (depending on the isolate) a white precipitation line was observed for positive WLA organisms (Figure 2-15).

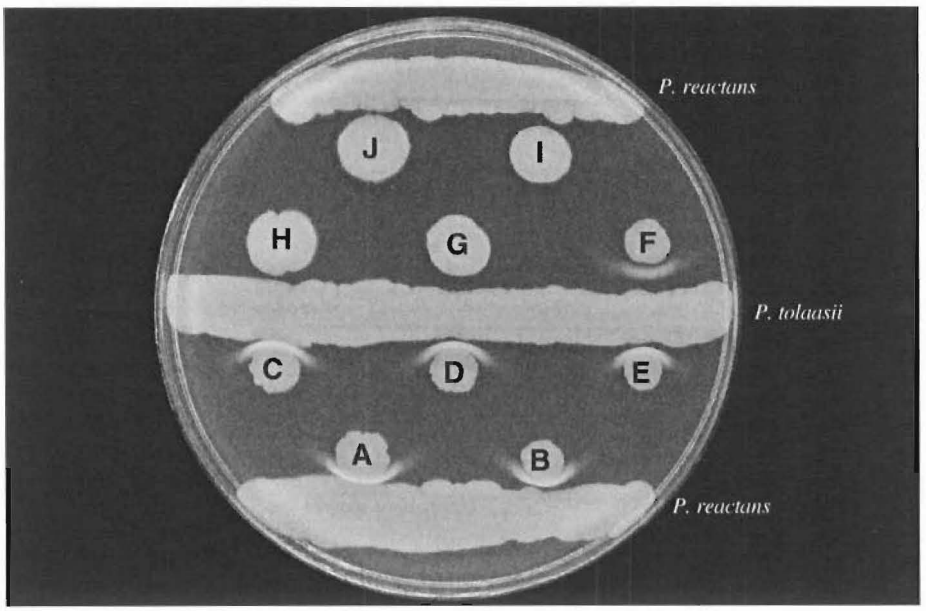

Figure 2-15 White line in agar (WLA) assay (Wong and Preece, 1979) of BCOs grown in close proximity to indicator strains. Shown is the characteristic white precipitate formed when lipodepsipeptide is produced by two reactor organisms. Selected isolates are: (A) NZ027 and (B) NZ032 in reaction to *P. reactans* NCPPB 1311. (C) NZ009 (D), NZ052 (E), NZ060 and (F) NZ097 in reaction to *P. tolaasii* NCPPB 2192. Negative isolates shown are: (G) NZ103 and (H) NZ011 in non-reaction to *P. tolaasii* NCPPB 2192 and (I) NZ043 and (J) NZ 047 in non-reaction to *P. reactans* NCPPB 1311.

Eight of the 33 BCO isolates (NZ009, NZ052, NZ060, NZ007, NZ065, NZ097, NZ101 and NZ096) produced positive white-line-in-agar (WLA+) reactions with *P. tolaasii*, consistent with the type strain of *P. reactans* (Table 2-2). Two isolates (NZ 027 and NZ 032) produced a WLA+ with *P. reactans*, as would be expected of *P. tolaasii* isolates.

2.12 API 20 NE biochemical analysis of BCO isolates

Tentative species identification of 33 BCO isolates was initially based on API 20 NE biochemical analysis carried out for initial selection. All purified 33 isolates were subjected to API 20 NE analysis to validate their belonging to the genus *Pseudomonas* (Table 2-2) and any isolates identified not to be pseudomonads were discarded. The API 20 NE micro-method for the identification of non-fastidious Gram-negative rods, using 8 conventional biochemical and 12 carbohydrate assimilation tests, was performed as described by the manufacturer (Bio Meriuex). The numerical profiles obtained from pseudomonad strains were compared to the profiles stored in the 1999 Analytical Profile Index Software database (Bio Meriuex). Comparison of API 20 NE biotypes obtained for the 33 BCOs to

the corresponding API 20 NE database, identified NZ112 and NZ101 as *P. putida*, NZ097 as *Burkholderia cepacia*, and the remaining 30 BCO isolates as *P. fluorescens* (Table 2-2).

Although these 30 isolates were assigned *P. fluorescens* speciation using profile assignment, results of the biochemical assimilation amongst these 30 *P. fluorescens* showed that apart from the utilisation of certain sugars[1] and positive oxidase test[2], clear differences amongst the other tests were observed. However, even given these profile differences, it became obvious that further discrimination of all 33 BCO isolates was necessary due to the inability of the API 20 NE test to discriminate between isolates and assign correct speciation. For instance, the type strain of *P. tolaasii* (NCPPB 2192T) could not be distinguished from *P. fluorescens*.

2.13 Phylogenetic characterization of BCO isolates

As the API20 NE biochemical assimilation analyses gave no resolution to species identity, the genetic relatedness of the 33 BCOs was determined by sequencing the 16S rRNA gene of each strain and comparing them with previously reported 16S rRNA gene sequences of members in the genus *Pseudomonas*. Nucleotide sequence analysis of the 16S rRNA gene was used to compare pseudomonads in this study because it is considered an effective method for defining prokaryotic genotypic relatedness and resolving taxonomic identities (Fox, *et al.*, 1980, Moore, *et al.*, 1996, Head, *et al.*, 1998).

Sequencing of the 16S rRNA gene

PCR amplification using oligonucleotide primers U16A and U16B (Wang and Wang, 1996) were used to amplify the nearly complete 16S rRNA gene (ca. 1480 bp depending on species) from BCO isolates and type *Pseudomonas* strains: *P. tolaasii* NCPPB2192T, *P. gingeri* NCPPB3147T and *P. reactans* NCPPB1311 using standard PCR conditions (Appendix I(iv)b). Direct nucleotide sequencing (Appendix I(v)) of this gene was achieved using oligonucleotide primers[3] U16A, U16B, and F357, F945, R1087 and R518 (Lane, 1991) (Figure 2-16) to ensure provision of overlapping sequence analyses.

[1] Rhamnose, melibiose, amygdalin.
[2] Indicates the presence of cytochrome-oxidase.
[3] Nucleotide sequences for respective primers are given in Appendix VI.

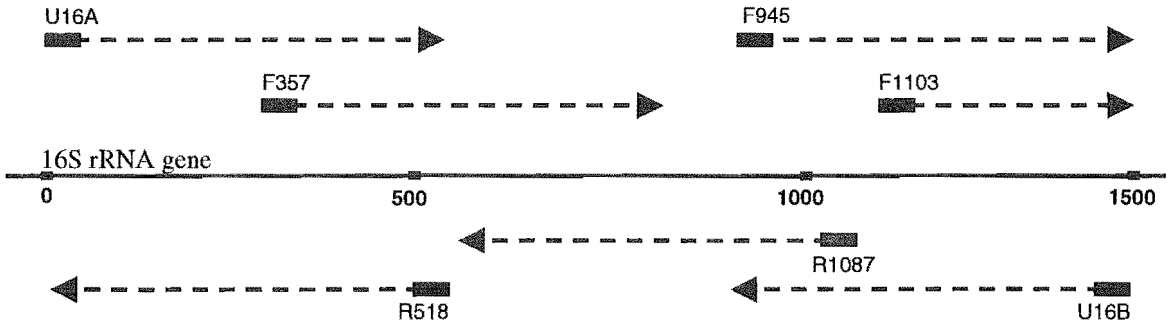

Figure 2-16 Schematic representation of oligonucleotide primers (Lane, 1991) used for sequence analysis of the near complete 16S rRNA gene of pseudomonads in this study. Boxes indicate priming locations and dashed lines represent direction of nucleotide sequencing.

All 16S rRNA gene sequences analyzed in this study were confirmed by determining contiguous overlapping sequences of PCR-sequenced DNA using Sequencher™ 3.0 sequence analysis software package (Gene Codes Corporation) and any ambiguous nucleotides were re-sequenced to ensure accuracy. All confirmed 16S rRNA sequences have been deposited with GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers listed in Table 2-2.

Phylogenetic analysis

Twenty-two further *Pseudomonas* 16S rRNA sequences (Table 2-1) were obtained from GenBank to allow comparison of BCO speciation. These selected 22 pseudomonads are validated type strains of the respective *Pseudomonas* species (Moore, *et al.*, 1996).

Table 2-1 Twenty-two validly described species of the genus *Pseudomonas* (sensu stricto) (Moore, *et al.*, 1996) used in 16S rRNA gene phylogenetic analysis. These 16S rRNA gene sequences were retrieved from GenBank using accession numbers below.

Bacterial isolate	Strain designation [a]	GenBank Accession
P. aeruginosa	LMG 1242T[b]	Z76651
P. agarici	LMG 2112T	Z76652
P. alcaligenes	LMG 1224T	Z76653
P. amygdali	LMG 2123T	Z76654
P. asplenii	LMG 2137T	Z76655
P. aureofaciens	DSM 6698T	Z76656
P. balearica	DSM 6083T	U26418
P. chlororaphis	LMG 5004T	Z76657
P. cichorii	LMG 2162T	Z76658
P. citronellolis	DSM 50332T	Z76659
P. coronafaciens	LMG 13190T	Z76660
P. ficuserectae	LMG 5694T	Z76661
P. flavescens	NCPPB 3063T	U01916
P. fluorescens biotype A	DSM 50090T	Z76662
P. marginalis pv. *marginalis*	LMG 2210T	Z76663
P. mendocina	LMG 1223T	Z76664
P. oleovorans	DSM 1045T	Z76665
P. putida biotype A	DSM 291T	Z76667
P. stutzeri	CCUG 11256T	U26262
P. syringae pv. *syringae*	LMG 1247t1T	Z76669
P. tolaasii	LMG 2342T	Z76670
P. viridiflava	LMG 2352T	Z76671
Acinetobacter calcoaceticus	ATCC 23055	Z93434

a ATCC, American Type Culture Collection, Rockville, Maryland, USA; DSM, Deutsche Sammlung von Mikro-organismen, Gottingen, Germany; LMG, Laboratorium voor Microbiologie en Genetica, Rijksuniversiteit, Gent, Belgium; IAM, Institute of Applied Microbiology, Tokyo, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK.

b T = Type strain.

The 16S rRNA gene nucleotide sequences obtained in this study were aligned with these validated 22 pseudomonad 16S rRNA sequences (Table 2-1) using the nucleotide alignment software Clustal W (Thompson, *et al.*, 1994). Phylogenetic trees were constructed with neighbor joining (Saitou and Nei, 1987) and evolutionary distances calculated according to (Jukes and Cantor, 1969) using the software package Treecon for Windows version 1.3b (Van de Peer and De Wachter, 1994). Bootstrap analysis (Felsenstein, 1985) was carried out using 500 replicates. *Acinetobacter calcoaceticus* ATCC 23055 was included for single sequence (forced) outgroup rooting of the tree. A resulting dendrogram was created and based on the extensive study by Moore *et al.* (1996), two major intrageneric clusters (*P. aeruginosa* and the *P. fluorescens* intrageneric cluster) and five evolutionary lineages were defined (*P. fluorescens* lineage, *P. cichorii* lineage, *P. syringae* lineage, *P. putida* lineage and *P. agarici* lineage) (Figure 2-17).

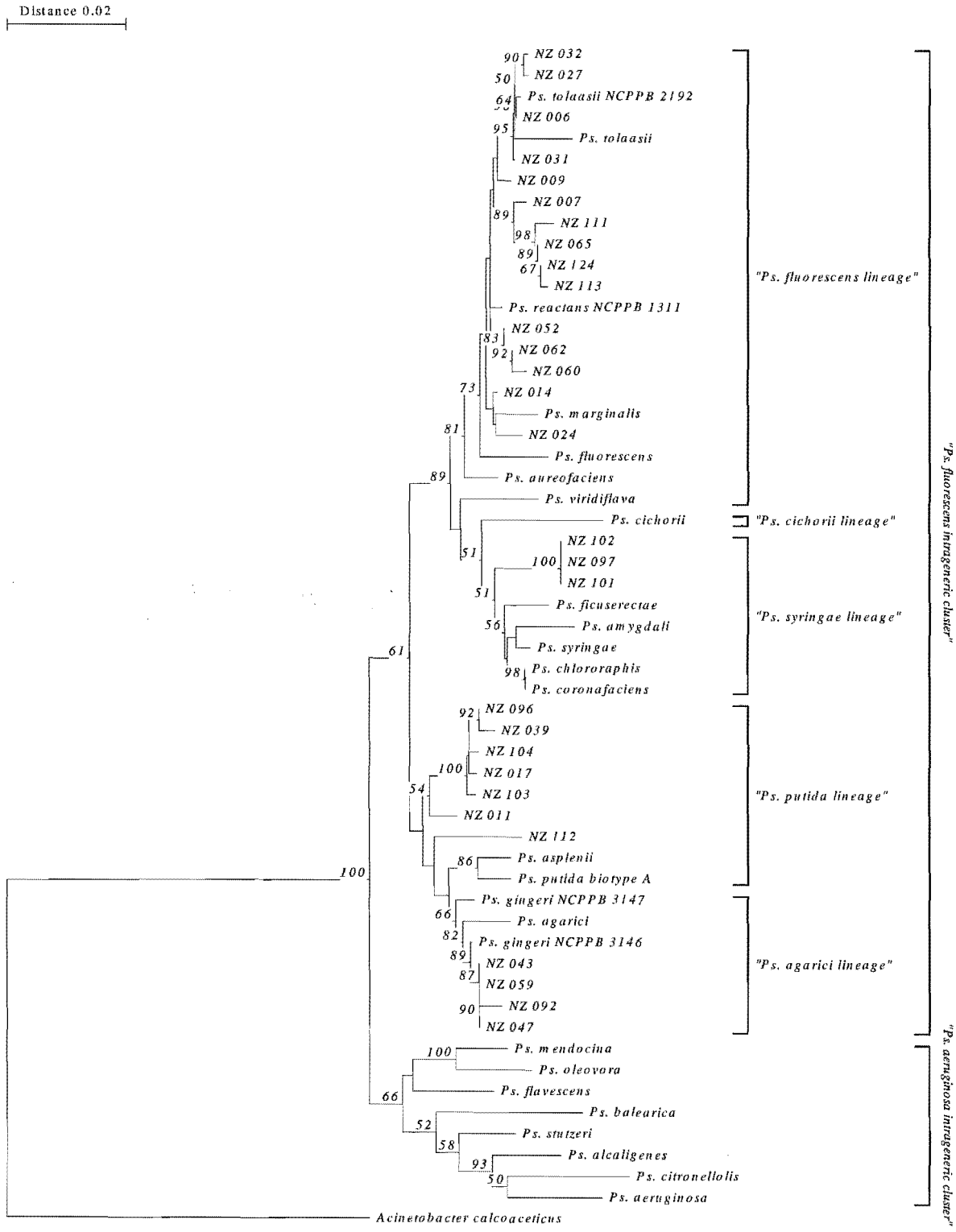

Figure 2-17 The inferred phylogenetic relationships between the 33 BCO isolates from this study and 22 validly described members of the genus *Pseudomonas* (sensu stricto). Evolutionary distances were determined with pairwise dissimilarities of the 16S rRNA gene sequences and the dendrogram was generated using the neighbor-joining algorithm. Two major intrageneric clusters and five evolutionary lineages are defined as described (Moore, *et al.*, 1996). Bootstrap proportions of confidence are represented as percentages for those branchings with values greater than 50%.

All 33 BCO isolates from this study clustered within the "*P. fluorescens* intrageneric cluster", not within the "*P. aeruginosa* intrageneric cluster" (as defined by (Moore, *et al.*, 1996)). Only four BCO isolates (NZ043, NZ059, NZ092 and NZ047) were observed to group closely with the previously identified typed strain of *P. gingeri* NCPPB 3147T within the "*P. agarici* lineage" (Figure 2-17). The remaining 29 isolates were distributed throughout the "*P. fluorescens* intrageneric cluster", with 18 (the majority) of isolates falling in the *P. fluorescens* lineage, eight within the *P. putida* lineage (five forming a tight cluster) and three within the *P. syringae* lineage. Consistent with the WLA+, NZ027 and NZ032 grouped closely with *P. tolaasii*, strongly suggesting identity as *P. tolaasii*. Isolates that reacted with *P. tolaasii* (therefore classed as WLIP organisms, including *P. reactans* (Mortishire-Smith, *et al.*, 1991)) were located mainly in the *P. fluorescens* lineage, although there was also intrageneric clustering within the *P. syringae* and *P. putida* lineages as well. Isolates grouping with *P. gingeri* NCPPB 3147T (NZ043, NZ059, NZ092 and NZ047) exhibited discolourations consistent with 'ginger blotch' (defined in this study as B4 - B6 based on a single color scale factor either side of the B5 discolouration caused by *P. gingeri* NCPPB 3147T). However, B4 - B6 discolourations were also observed in BCO isolates in the *P. fluorescens* lineage (NZ006, B6; NZ031, B4; NZ007, B4; NZ060, B4), the *P. syringae* lineage (NZ101, B6) and the *P. putida* lineage (NZ011, B6) (Figure 2-17).

2.14 Summary of BCO analysis

Table 2-2 New Zealand BCO isolates and reference pseudomonad strains used in this study. For discussion purposes, isolates have been ordered as phylogenetically defined in Figure 2-17

Designation	Farm[a]	Assay[c]	Com[d]	WL(R)[e]	WL(T)[f]	Biotype[g]	Accession No.[h]
NZ 032	A	B9	B	+	-	0156555	AF320995
NZ 027	B	B9	B	+	-	0156555	AF320994
NZ 006	A	B6	-	-	-	0156555	AY014800
P. tolaasii 2192Ti	N[b]	B9	B	+	-	0156555	AF320988
NZ 031	C	B4	-	-	-	0177575	AY014807
NZ 009	A	B3	-	-	+	1357555	AY014802
P. reactans 1311	N	B2	-	-	+	0357555	AF320987
NZ 014	A	B3	D	-	-	1147555	AY014804
NZ 024	A	B3	D	-	-	1147455	AY014806
NZ 052	A	B9	-	-	+	0157575	AY014811
NZ 062	A	B9	Y	-	-	0157575	AY014814
NZ 060	A	B4	B	-	+	0157575	AY014813
NZ 007	A	B4	-	-	+	0557555	AY014801
NZ 081	B	B1	-	-	-	0757555	AF388206
NZ 111	E	B9	D	-	-	0157577	AY014825
NZ 065	B	B3	D	-	+	0757555	AY014815
NZ 124	C	B8	B	-	-	0157555	AY014829
NZ 066	B	B1	-	-	-	0157555	AF388209
NZ 113	E	B3	-	-	-	0357555	AY014827
NZ 064	A	B1	-	-	-	0357555	AF388208
NZ 102	A	B3	B	-	-	0357555	AY014820
NZ 097	A	B3	-	-	+	4156577	AY014818
NZ 101	A	B6	-	-	+	0146657	AY014819
NZ 096	A	B2	-	-	+	0157555	AY014817
NZ 039	D	B3	Y	-	-	0157555	AY014808
NZ 104	A	B3	-	-	-	0157555	AY014822
NZ 017	A	B3	D	-	-	1357555	AY014805
NZ 103	A	B9	B	-	-	0157555	AY014821
NZ 099	A	B1	-	-	-	0357555	AF388207
NZ 011	A	B6	-	-	-	0157555	AY014803
NZ 112	E	B3	-	-	-	4140457	AY014826
P. gingeri 3147T	N	B5	Y	-	-	0356555	AF320991
NZ 043	B	B5	Y	-	-	0157555	AY014809
NZ 059	A	B6	B	-	-	0156555	AY014812
NZ 092	A	B5	-	-	-	0156555	AY014816
NZ 047	B	B5	Y	-	-	0156565	AY014810

[a] topographically distinct farm locations designated A-D (A, major, B-D, minor) from which pseudomonads were isolated, E, milk processing plant isolates (Reid, 1997).
[b] N = reference strains obtained from National Collection of Plant Pathogenic Bacteria, Harpenden, UK.
[c] discolouration of mushroom cube bioassay (11) after 48 hr where B = blotch.
[d] comments where: D = tissue degradation; Y = yellowing of filter paper; B = browning of filter paper.
[e] white line reaction with *P. reactans* NCPPB 1311.
[f] white line reaction with *P. tolaasii* NCPPB 2192T.
[g] API 20 NE numerical profile obtained.
[h] accession numbers of 16S rRNA nucleotide sequences obtained in this study and stored in GenBank.
[i] T = recognized type strain of species.

DISCUSSION

Previous studies addressing the relatedness of pathogenic pseudomonads that cause disease on cultivated *A. bisporus* are well documented and from these, *P. tolaasii* has been well characterized. However, *P. gingeri* has received limited attention since it was first described by (Wong, *et al.*, 1982).

Initiated by costly New Zealand outbreaks of ginger discolourations of *A. bisporus*, both on beds and in post-harvest, characterization of the causal organism(s) of 'ginger blotch' in New Zealand mushroom farms was sought. Observation of pseudomonads with variable colony morphology and growth patterns isolated from mushrooms exhibiting 'ginger blotch' symptoms questioned whether 'ginger blotch' of *A. bisporus* is caused by a homogeneous species previously described as *P. gingeri*.

2.14.1 Isolation of pseudomonads using Gould's medium

Initially, 95 pseudomonads associated with discolourations of *A. bisporus* were isolated from mushroom farms throughout New Zealand. Isolation using Gould's S1 agar (Gould, *et al.*, 1985) proved an effective media for *Pseudomonas* selection as API20 NE and 16S rRNA analyses confirmed that all 33 refined isolates belonged to the genus *Pseudomonas*. Furthermore, a good representation of *Pseudomonas* diversity was observed with Gould's media even though evidence exists that choice of media has an important role in determining the degree of *Pseudomonas* diversity recovery (Sorheim, *et al.*, 1989, Aagot, *et al.*, 2001).

It is acknowledged that only 'culturable' organisms defined by the culturing parameters used in this study were recovered. It has been estimated that only 1-5% of total bacterial populations can be readily cultivated using currently described media (Olsen and Bakken, 1987) and microbial diversity is approximately 170x times higher when represented by molecular methods (Torsvik, *et al.*, 1990). However, it was deemed that because pseudomonads are ubiquitous and frequently isolated (Palleroni, 1984) a high representation of pseudomonad species would be obtained from the mushroom environment in this study. Furthermore, this study was aimed as a preliminary investigation to determine the extent of pseudomonad diversity, not to characterise *all* pseudomonads able to cause blotch.

2.14.2 Cube bioassay results

Using chromametric measurements, variations of discolouration of *A. bisporus* tissue has been previously established for different pseudomonads (Soler-Rivas, *et al.*, 2000) in which *P. tolaasii* was shown to evoke a specific color change, while *P. gingeri* and *P. reactans* induced discolourations that were different to each other and to *P. tolaasii*. In order to determine pathogenic potential of the 33 BCOs in this study, the ability of each isolate to discolor and/or damage *A. bisporus* tissue was

assessed in bioassay with comparison to these reference strains (Table 2-2). Bioassay results of the initial pool of 95 isolates from around New Zealand showed 32/95 (33%) to cause 'ginger blotch' discolourations (B4-B6). However, the remaining 63 (67%) isolates were also observed to cause different degrees of *A. bisporus* discolouration (B1-B3 and B7-B9) and for this reason, the original focus of this study to characterise 'ginger blotch' was diverted to characterising all blotches of *A. bisporus* so as to ascertain the degree of diversity amongst pseudomonad species that were capable of causing blotch diseases.

For this purpose, 33 pseudomonad isolates were selected because they caused varying discolourations of *A. bisporus* tissue in bioassay but had distinguishing REP-PCR profiles from one another. In addition, three milk isolates were included in the 33 chosen for the genetic analysis because they also caused blotch discolourations of *A. bisporus*. As the primary focus of this section of study was to resolve the different types of pseudomonads able to cause blotch, no data was collected to determine the population dynamics of these 33 BCO isolates throughout New Zealand or to identify predominant BCO strains in any one farm.

2.14.3 Difficulty in fulfilling Koch's postulate

When studying diseases of cultivated mushrooms, it is difficult to fulfill Koch's postulate of a particular organism. Reinoculation of a suspected disease-causing organism into a mushroom farm is impractical for a number of reasons. Firstly, the non-repeatable variation in both biotic- and abiotic factors (e.g. starting materials, environmental conditions and microbial contamination) within a mushroom farm is very difficult, if not impossible, to replicate from trial-to-trial. Secondly, mushroom growers are not forthcoming in letting a suspected disease-causing agent onto their mushroom farm and be inoculated into a commercial crop that could result in crop loss, decline in crop quality, and have the potential to establish and spread. Therefore, in this study within New Zealand, fulfilling Koch's postulate was not carried out. However, throughout the world mushroom growing sheds identical to those used in commercial production are established in order to carry out such disease introduction and crop effect/yield studies. Two such farms visited during this study were Horticulture Research International (HRI), Wellesbourne, England, and Queens University, Belfast, Northern Ireland. Unfortunately, it was impractical to use these facilities during this thesis and therefore it was necessary to establish a repeatable accurate *in vitro* bioassay for the determination of blotch causing ability of the BCOs isolated in this study.

It is also acknowledged that mushroom cube bioassays do not replicate the intact mushroom cap surface and therefore, may not give representation of *in situ* blotch formation. However, known blotch forming organisms from previous studies have been shown to cause discolouration in such tissue cube bioassays (Gandy, 1967, Wong and Preece, 1979) and therefore, in the scope of this study, this type of *in vitro* bioassay was deemed sufficient to determine and enable comparison of blotch discolourations amongst BCOs.

2.14.4 Limitations of phenotypic assays used in this study

Many studies are undertaken because they have an easy phenotype to observe, and alterations to this phenotype can be easily monitored. An example of such a phenotype is that of *P. tolaasii* tolaasin production using the WLA assay that gives a clearly visible white-line precipitate phenotype (Figure 2-8). Such a phenotype enables numerous mutants to be screened with effective 'yes/no' answers to phenotype production. However, the blotch bioassay used in this study did not provide such a straightforward 'yes/no' answer due to the compositional variance in mushroom tissue discolouration observed amongst different *A. bisporus* sources (Leeuwen and Wichers, 1999). This was circumvented by performing numerous independent replicates of bioassays (as discussed below).

2.14.5 Development of repeatable mushroom cube bioassays

Bioassays in this study were carried out in triplicate using different sources of *A. bisporus* tissue in each replicate. This was to ensure accurate results of *A. bisporus* tissue discolouration, as amounts of tyrosinase (Jolivet, *et al.*, 1995) have been shown to vary depending on spatial location and developmental stages of the mushroom (Leeuwen and Wichers, 1999) (discussed in Chapter 1.2.2). Furthermore, differences in susceptibility of *A. bisporus* to bacterial blotch are also influenced by parameters of *A. bisporus* strain type (Moquet, *et al.*, 1998) and parameters of the mushroom cultivation with compost quality being of prime importance (Mamoun, *et al.*, 2000).

Known concentrations (ca. $1x10^7$ $cfu.ml^{-1}$) of pseudomonad isolates were inoculated in each bioassay to ensure equal bacterial loading on each mushroom cube. This was to facilitate direct comparison between results as different bacterial loadings on caps are thought to influence the development of the discolourations (Soler-Rivas, *et al.*, 2000). Discolourations observed in these mushroom cube bioassays could therefore, be assumed to be the result of individual strain virulence factors alone. It was noted that different isolates formed discolourations more rapidly than others. This may have been a result of faster multiplying isolates, although the degree of replication was not taken into account in this study because it was found that once discolouration had occurred, it remained constant. Most discolouration occurred within 48 hr, and therefore, bioassay results were ultimately determined after 48hr.

2.14.6 Biochemical analysis using API 20 NE

Species-similarity of the 33 BCO isolates was initially sought by biochemical analysis. While the carbon assimilation tests and production of enzyme intermediates included in the API 20 NE strip do not effectively discriminate between isolates of *P. gingeri*, *P. tolaasii*, *P. reactans* or between biovars of *P. fluorescens* isolates, clear biotype differences between many of the 33 BCO isolates were observed (Table 2-2). For this reason, API 20 NE strips were useful in determining differences in biochemical phenotypes of the BCO isolates, but did not facilitate species identification.

2.14.7 Phylogenetic relationship analysis overview

To determine the phylogenetic relationship of the 33 selected BCO isolates, comparison of nucleotide sequences of the 16S rRNA gene was used in this study; as it is considered an effective method for defining prokaryotic genotypic relatedness and resolving taxonomic identities (Fox, *et al.*, 1980, Laguerre, *et al.*, 1994, Moore, *et al.*, 1996, Head, *et al.*, 1998). The overall topology of the dendrogram derived from 16S rRNA in this study (Figure 2-17) is in agreement with the intrageneric structure determined previously for the genus *Pseudomonas* (Moore, *et al.*, 1996). The 33 BCO isolates distributed throughout the "*P. fluorescens* intrageneric cluster", supporting previous studies (Soler-Rivas, *et al.*, 2000) in which distantly related pseudomonads (*P. tolaasii*, *P. gingeri* and *P. reactans*) caused different degrees of discolouration. However, this study further demonstrates the vast extent of the diversity of pseudomonads capable of inducing discolouration of *A. bisporus*. These results also show that the same degree of discolouration may be caused by dissimilar species of pseudomonads, suggesting that the factor(s) causative of inducing a particular discolouration of *A. bisporus* are not exclusive to a particular pseudomonad species.

2.14.8 Competition leads to diversity

As pseudomonads are arguably the most diverse and ecologically significant group of bacteria (Palleroni, 1993), observation of the degree of diversity within this study in the mushroom niche is not altogether unexpected. In a series of now classic experiments using test tube populations of *Paramecium*, it was shown that two species competing for the same niche could not coexist because one would drive the other to extinction (*i.e.* the niche-exclusion principle (Gause, 1934)). However, this applied for environments that lack spatial or temporal heterogeneity (*i.e.* is homogeneous) and therefore provides only a single niche. Bacterial niches provided within mushroom cultivation provide a diverse source of environmental niches, each with different nutritional composition and therefore, are far from homogeneous; so it is not unexpected that much *Pseudomonas* diversity exists.

As the density of any given bacterial genotype growing in a simple structured environment increases, growth eventually becomes limited due to depletion of resources such as oxygen and nutrients. At this stage competition becomes intense and selection will favour any mutant that can gain access to limiting resources. The most favoured genotypes will be those that occupy niches that are different to that occupied by the dominant genotype. Having diverged, selection will continue to favour mutants that show increased degrees of niche specialization so that eventually there will be minimal niche overlap among genotypes (Spiers, *et al.*, 2000). This can be envisaged in the mushroom farm environment where there is an abundance of nutrients initially, but given the exponential growth of bacteria, these nutrients may soon deplete. This is when adaptive mutations for the utilization of alternative nutrient sources would be advantageous. If a bacterium had the ability to colonise mushroom hyphae, then it would be maximizing an opportunity in an environmental niche

that provides many nutrients (Samson, *et al.*, 1986). Furthermore, if a bacterium has the ability to produce extracellular compounds to obtain more nutrients from the mushroom mycelium, then this may provide it with an evolutionary advantage. This could account for the great diversity of blotch causing pseudomonads found in this study.

2.14.9 Advent of molecular techniques

Numerous species within the genus *Pseudomonas* have been classified (Stanier, *et al.*, 1966, Palleroni, 1984, Palleroni, 1986) and many new species continue to be identified by methods revised in response to advances in DNA technology. These include: DNA-DNA hybridization (Wayne, *et al.*, 1987), gene sequence analysis of 16S rRNA (Laguerre, *et al.*, 1994, Moore, *et al.*, 1996) and *gyrB* and *rpoD* (Yamamoto, *et al.*, 2000). Such methods have aided taxonomic resolution, but as this study has shown, they may also introduce discrepancies between phenotypic and genotypic analyses. For example, Moore *et al.* (Moore, *et al.*, 1996) demonstrated seven, phenotypically indistinguishable, genomovars of *P. stutzeri* that contain up to six nucleotide differences within the 16S rRNA gene and Yamamoto *et al.* (Yamamoto, *et al.*, 2000) also observed that many phenotypic traits of pseudomonad species did not reflect their phylogenetic relationships (e.g. *P. corrugata* was observed in the *'P. fluorescens* complex' and *P. amygdali* in the *'P. syringae* complex'). These discrepancies between bacterial species will continue to arise until criteria are adequately defined for universal assignment and definition of a given species (a species concept) (Mortishire-Smith, *et al.*, 1991).

2.14.10 Species concept

One reason why evolutionary biology sometimes fails to gain the respect of other scientists is because of its endless debates over questions that focus on semantics. The most famous (or infamous) of these involves the definition of a species. Most biologists think of species in terms of the biological species concept, but with the advent of molecular techniques allowing highly detailed examination of individuals, the species concept necessitates revision (Noor, 2002). Furthermore, defining a bacterial species concept in intrinsically more complex given the recognition of the significance that recombinational events contributes to bacterial population genetics (Maynard Smith, 1995).

In light of the limitation of assigning speciation to the 33 isolated pseudomonads, 16S rRNA gene analyses carried out in this study were intended to: a) provide an indication of BCO species similarity; and 2) enable comparisons amongst BCOs only. The results in this study cannot account for the acquisition of genes and accessory genetic elements (plasmids, transposons, integrons and phages) by lateral gene transfer (Ochman, *et al.*, 2000, Spiers, *et al.*, 2000), classical 'spontaneous' mutation (Levin and Bergstrom, 2000) and recombination (Haubold and Rainey, 1996, Kiewitz and Tummier, 2000) - all of which are important sources of bacterial evolution and species diversity.

2.14.11 Correlation of 'ginger blotch' with BCOs

With the aim of identifying ginger blotch causing organisms, there was still much interest in elucidating BCO isolates capable of causing discolourations that are comparable to *P. gingeri* NCPPB 3147T (B4-B6). Four BCO isolates were observed to group closely with *P. gingeri* reference strains based on the 16S rRNA phylogenetic analysis (NZ043, NZ059, NZ092 and NZ047). These isolates were also observed to cause B4-B6 discolourations, suggesting that isolates closely related to *P. gingeri* have the ability to induce *A. bisporus* discolouration consistent with 'ginger blotch'. However, a further six BCO isolates causing B4-B6 discolourations were distributed within the *P. fluorescens* lineage, the *P. syringae* lineage, and the *P. putida* lineage. This result suggests the organism previously described as "*P. gingeri*" is not solely responsible for 'ginger blotch' of *A. bisporus* and the disease can be caused by a number of different pseudomonads. This single result within this study has important implications on future classification of *P. gingeri* given that a major criteria of its identification is the ability to cause ginger discolouration of *A. bisporus* tissue.

2.14.12 Correlation of lipodepsipeptide (LDP) and blotch discolouration

As production of lipodepsipeptide (LDP) can be a pathogenicity factor associated with *A. bisporus* discolouration (Wells, *et al.*, 1996, Soler-Rivas, *et al.*, 1999a), each BCO isolate was assayed for its ability to secrete an LDP capable of forming a white line in agar (WLA) precipitate with either *P. reactans* or *P. tolaasii*. Observation of eight BCO isolates producing WLA+ reactions with *P. tolaasii* suggested they produced LDP similar to the white line inducing principle (WLIP) described previously (Mortishire-Smith, *et al.*, 1991). Phylogenetic analysis of these eight BCO isolates (Figure 2-17) again revealed no commonality of LDP production with either blotch discolouration (Table 2-2) or species identity (Figure 2-17). For example, although NZ 052 and NZ 062 grouped closely together, only NZ 052 exhibited a WLA+ with *P. tolaasii*. Isolates NZ 096, NZ 103 and NZ 104 also clustered tightly, yet exhibited different bioassay discolourations, and of the three, only NZ 096 was WLA+ with *P. tolaasii*. Although these two groupings exhibit high phylogenetic similarity, they display quite different pathogenicity (including the presence of the potential virulence factor WLIP) that may suggest that these isolates have acquired traits responsible for differing degrees of *A. bisporus* discolourations. Studies have revealed that horizontal transfer and recombination of virulence genes plays a major role in generating genetic diversity amongst bacterial species (Kehoe, *et al.*, 1996) and horizontal gene acquisition could also be an explanation of why highly related BCO isolates (based on 16S rRNA) in this study have different virulence potentials. Furthermore, it must be reemphasised that 16S rRNA analysis does not give account of important sources of bacterial species diversity discussed earlier (*i.e.* plasmids, transposons, integrons and phages, mutation and recombination (Spiers, *et al.*, 2000)).

2.14.13 Extracellular enzymes

Because LDPs could not be correlated with any given blotch phenotype, other mechanisms involved in blotch discolouration were considered. Most members of the genus *Pseudomonas* produce active extracellular enzymes that have been associated with plant disease, including proteinases and lipases. *P. tolaasii* was found to produce a proteinase very similar to those secreted by other *Pseudomonas* species (Fairbairn and Law, 1986) that, despite the effect of this proteinase in mushroom infection remaining unknown, may facilitate the damage caused in the mushroom (Baral, *et al.*, 1995). Lipases have also been shown to facilitate bacterial infections by disrupting host membranes and *P. tolaasii*, like many other pathogenic pseudomonads, produces an extracellular heat-stable monomeric metallo-lipase however, its involvement in mushroom infection is unresolved (Baral and Fox, 1997). Like *P. tolaasii*, it may be assumed that the BCO isolates in this study produce extracellular enzymes, including proteinases and lipases, which are likely to be involved in the discolouration of *A. bisporus*. The degrees of discolouration observed in this study may be a result of different combinations of extracellular enzymes produced by the BCO isolates facilitating different enzymatic activation of *A. bisporus* tissue. The production of cytochrome-oxidase[1] was considered in that it may mimic the *A. bisporus* produced polyphenol oxidases (PPOs)[2] involved in browning. However, because all isolates, able to cause blotch or not, had cytochrome-oxidase present and therefore, it was not considered to be a major component of discolouration. However, identification of such putative pathogenicity determinants involved in blotch discolourations are further investigated and discussed in Chapter 4.

2.14.14 Consistency of discolorations observed in *A. bisporus* bioassays

P. tolaasii has routinely been distinguished from other pseudomonads by its ability to cause dark brown discolourations on mushrooms and by a positive WLA with *P. reactans* (Rhodes, 1959, Wong and Preece, 1979, Zarkower, *et al.*, 1983, Palleroni, 1984, Janse, *et al.*, 1992). NZ 027 and NZ 032 were included in this study and the initial assumption that they were *P. tolaasii* isolates based on WLA+ with *P. reactans* and B9 discolourations[3]. This was further supported by genetic identity with other *P. tolaasii* isolates. However, as observed with *P. gingeri* and 'ginger blotch' discolourations, B8–B9 BCO isolates showed little phylogenetic relatedness to *P. tolaasii* (with the exception of NZ 006) and were observed to distribute widely throughout the "*P. fluorescens* intrageneric cluster". As B8–B9 BCO isolates gave WLA- with *P. reactans* (except NZ 027 and NZ 032) it was assumed that browning was not due to tolaasin production, but most likely due to an extracellularly produced factor(s) that: (1) causes tyrosinase activation and production of brown melanins similar to tolaasin

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[1] Determined as part of the API 20 NE biochemical analysis in Section 2.12.
[2] Discussed in Chapter 1.2.
[3] Note that the validity of these tests is questioned by the results presented in Chapter 3.

(Burton, 1988a, Soulier, *et al.*, 1993, Jolivet, *et al.*, 1995); or (2) reduces enzymatic activity in *A. bisporus* tissue due to protease activity (Soler-Rivas, *et al.*, 2000). Proteases have been known to degrade tyrosinases (Burton, *et al.*, 1993) and a protease from *P. tolaasii* has been isolated and is speculated to facilitate damage to the mushroom (Baral, *et al.*, 1995).

2.14.15 Inclusion of milk isolates to look at heterogeneity of blotch formation

Also included in this study were three pseudomonad isolates from a New Zealand milk factory environment (NZ 111, NZ 112 and NZ 113) (Reid, 1997). These milk-isolates were initially included to ascertain if blotch formation was the result of environmental adaptation of pseudomonads in the mushroom farm in response to selective pressure(s). It was shown that these three isolates caused discolouration of *A. bisporus* as efficiently as the BCO isolates from mushroom farms. This observation: (1) demonstrates that the ability to cause discolouration of *A. bisporus* is not a trait acquired for evolutionary survival within pseudomonads present in the mushroom environment; (2) further supports the previous discussions that the factor(s) causing blotch discolourations are likely to be a combination of extracellular enzymes common to many different pseudomonads; and (3) raises the question as to whether other bacterial species may also be capable of induction of blotch discolourations of *A. bisporus*.

2.14.16 What elicits blotch discolourations?

Given the evidence for a correlation between bacterial numbers and blotch discolouration[1], it is likely that 'something' within these high bacterial numbers causes the blotch discolouration. Furthermore, some bacteria have this 'something' and others don't, as evidenced by selected pseudomonads in this study that did not induce blotch discolourations, even at high bacterial loadings. This 'something' could be defined as an elicitor of blotch discolourations, or a putative pathogenicity determinant and is the focus of Chapter 4.

[1] Threshold values of *P. tolaasii* inoculation are discussed in Chapter 1.5.6.

CONCLUSIONS

The original aim of this study was to address the relatedness of pseudomonads capable of inducing 'ginger blotch' disease of *A. bisporus* within New Zealand mushroom farms. Results showed many different blotch discolorations and a high degree of diversity of pseudomonads that caused them, and as such, this study has confirmed previous reports that blotch disease may be caused by different species of pseudomonads. Furthermore, these results have identified three major findings: i) the diversity of pseudomonads capable of causing blotch discolourations of *A. bisporus* is considerably more extensive than previously thought; ii) the organism previously described as *P. gingeri* is not solely responsible for ginger discolourations of *A. bisporus* ('ginger blotch'); and iii) a particular blotch discolouration may be caused by more than a single pseudomonad species. These findings affect the future classification of *P. gingeri* because a major phenotypic characteristic of this species is its ability to induce a ginger discolouration of *A. bisporus*. Furthermore, considering many different pseudomonads are able to cause different degrees of blotch and that pseudomonads are ubiquitous within the mushroom farm, then it is likely that blotch will form given the right environmental conditions.

Moreover, the inclusion of three milk isolates that can efficiently cause blotch suggests that blotch may not necessarily be the result of acquired adaptation by pseudomonads in a mushroom farm niche, but could be: i) a defense response by the mushroom in response to the colonisation and multiplication of opportunistic bacteria on the cap surface; or ii) the eliciting factor for blotch may be common to many pseudomonads. Investigation of such elicitor components is the focus of Chapter 4.

The results in this chapter have implications for the control of blotch diseases of *A. bisporus* since a single causal organism cannot be targeted. As certain pseudomonads are considered beneficial in the commercial cultivation of *A. bisporus* (Rainey, 1989), the elimination of all pseudomonads from a farm environment is neither desirable nor practical. Therefore, blotch disease of *A. bisporus* may prove difficult to manage and continued research of BCO organisms may better resolve the commonality of virulence factors and the environmental conditions that promote disease.

Chapter 3

CHARACTERISATION OF *PSEUDOMONAS* NZI7

As has been demonstrated in Chapter 2, diverse species of the genus *Pseudomonas* have the ability to produce diverse blotch phenotypes. Presented in this chapter is a detailed study of a single *Pseudomonas* isolate from a major New Zealand mushroom farm, isolated in a separate survey to that of Chapter 2. In this particular survey, the distribution of *P. tolaasii* contamination sites on this farm was sought. Isolated fluorescent pseudomonads were screened for a characteristic marker of *P. tolaasii*, the lipodepsipeptide tolaasin, using the white-line-in-agar (WLA) assay. One isolate, *Pseudomonas* NZI7, produced a positive WLA assay result and caused brown lesions of *A. bisporus* comparable to those produced by *P. tolaasii*. However, genetic analysis suggested that *P. tolaasii* and NZI7 were genetically dissimilar and that NZI7 is closely related to *Pseudomonas syringae*. Nucleotide sequence analyses of a gene involved in tolaasin production indicate similar genes are present in both NZI7 and *P. tolaasii*. The identification of NZI7 is significant in that it identifies limitations to the specificity on the previously accepted phenotypic identification of *P. tolaasii* based on *A. bisporus* browning and positive WLA result.

INTRODUCTION

P. tolaasii causes brown blotch disease of commercial mushrooms and is the primary bacterial agent responsible for disease symptoms is the extracellular toxin, tolaasin (Brodey, *et al.*, 1991, Nutkins, *et al.*, 1991, Rainey, *et al.*, 1991). Although correlation is well established between environmental conditions and disease symptoms, there are still no effective strategies for control of brown blotch. Therefore, it was desirable to develop a method for the efficient identification of *P. tolaasii*. Such a test was developed from the observation that when *P. tolaasii* is cultured in close proximity to a second pseudomonad, *P. 'reactans'*, a white precipitate forms between the colonies. This was defined as the white-line in agar (WLA) assay (Wong and Preece, 1979) and the WLA was assessed to specifically detect *P. tolaasii* isolates within this thesis as an applied study of a local New Zealand mushroom farm.

3.1 Taxonomic classification of *P. tolaasii*

Although *P. tolaasii* is included in the 'Approved Lists of Bacterial Names' (Skerman, *et al.*, 1989), its validity as a distinct species and its place within the genus *Pseudomonas* have not been fully resolved. Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) includes *P. tolaasii* in *Pseudomonas* Section V but hybridisation experiments involving 23S rRNA-DNA place *P. tolaasii* in Section I of the pseudomonads (De Vos, *et al.*, 1985).

Similarity of *P. tolaasii* amongst other *Pseudomonas* species

Although conserved regions in the gene encoding the outer-membrane protein (OprF) of both *Pseudomonas fluorescens* and *P. tolaasii* support the close relatedness of these two species, *P. tolaasii* is distinguishable from *P. fluorescens* (De Mot, *et al.*, 1994). *P. tolaasii* homogeneity and differentiation from both *P. fluorescens* and *P. 'reactans'* was demonstrated by Goor et al. (1986) using substrate utilization tests, electrophoresis of soluble proteins, and DNA:DNA hybridization experiments. Goor et al. (1986) also identified several *P. tolaasii* strains that were WLA negative and non-pathogenic to mushrooms. Thorn and Akihiko (1996) addressed the species homogeneity of geographically diverse isolates of *P. tolaasii* using DNA restriction fragment length polymorphisms (RFLP) and partial sequence analysis of PCR-amplified 16S rRNA genes. These experiments indicated that isolates of *P. tolaasii* were highly similar to each other, but readily distinguished from *P. fluorescens* isolates from different environments. Further species grouping of *P. tolaasii* was demonstrated by Moore et al. (1996) based on comparison of the small subunit rRNA (16S rRNA) nucleotide sequences. This phylogenetic study included 24 validly-described species of the genus *Pseudomonas* (sensu stricto) and showed that the 16S rRNA hyper-variable region-2 was identical in

P. tolaasii isolates, but differed between *P. tolaasii* and all other species within the "*Pseudomonas fluorescens* lineage" (Moore, *et al.*, 1996).

3.2 White-line-in-agar (WLA) assay

The primary bacterial agent responsible for disease symptoms of *P. tolaasii* is the extracellular toxin, tolaasin (Brodey, *et al.*, 1991, Nutkins, *et al.*, 1991, Rainey, *et al.*, 1991). Tolaasin forms a dense white precipitate with the "white line inducing principle" (WLIP), a LDP produced by *P. 'reactans'* (Mortishire-Smith, *et al.*, 1991). Mutants of *P. tolaasii* that are defective in tolaasin production (discussed later) fail to form a white precipitate, thus strongly suggesting that tolaasin is the sole agent that reacts with WLIP (Rainey, *et al.*, 1993).

The physiological properties of *P. tolaasii* are similar to those of *P. fluorescens* and other fluorescent pseudomonads (Palleroni, 1984). However, *P. tolaasii* has routinely been distinguished from these organisms by its pathogenicity to mushrooms and by a positive WLA (Rhodes, 1959, Wong and Preece, 1979, Zarkower, *et al.*, 1983, Palleroni, 1984, Janse, *et al.*, 1992). To the knowledge of this author, there have been no published reports of a non-*P. tolaasii* isolate causing a positive WLA in reaction to *P. reactans*.

Apart from previously described mushroom pseudomonads discussed that produce white line inducing principle (WLIP) in response to *P. tolaasii*, *i.e. P. reactans* (Mortishire-Smith, *et al.*, 1991), there are also other reports of pseudomonads producing LDP. These include *P. corrugata* (corpeptin) (Emanuele, *et al.*, 1998), *P. fuscovaginae* (fuscopeptin) (Ballio, *et al.*, 1996), *P. syringae* pv. *syringae* (syringopeptins) (Ballio, *et al.*, 1991), and *P. fluorescens* (viscosin) (Laycock, *et al.*, 1991).

3.3 Tolaasin

Tolaasin is an extracellular compound produced by *P. tolaasii* that has been extensively studied and characterized (Soler-Rivas, *et al.*, 1999c). The observation that *P. tolaasii*, separated by a dialysis membrane, on a detached mushroom sporophore caused brown discolouration indicated for the first time the importance of extracellular compounds in the browning produced by the bacterium (Nair and Fahy, 1973). Browning of both mushroom tissue blocks and growing sporophores was also caused by cell-free culture filtrates. In addition, autoclaved cell-free culture filtrate was active in browning mushroom tissues over a range of pH 3 to 9. In conclusion, the possible occurrence of a heat stable toxin with a relatively low molecular weight (because it could pass through a membrane with a pore radius less than 20 A) was proposed. The toxin could also produce lesions on the leaves of wheat and inhibit of growth of wheat coleoptiles and roots (Malcom, 1981), suggesting it was not host specific.

3.3.1 Tolaasin production

Accounts of tolaasin production vary from author to author. The cell-density of *P. tolaasii* was found to increase during the log-phase of growth from 4-16 hrs, and a corresponding increase in browning of mushroom tissue was also observed between 4-12 hours, but no further browning increased beyond the log-phase into the stationary phase of bacterial growth (Nair and Fahy, 1973). These findings indicated that the toxin was produced during log-phase of bacterial growth, starting at the early part of exponential growth and ceasing during late exponential growth.

More recently, a different result was observed where an increase in the amount of tolaasin was noted during the stationary phase (Rainey, *et al.*, 1991). A sudden decrease in viable cell counts coincided with the onset of tolaasin production (later, (Baral, *et al.*, 1995) did not observe this decrease). Tolaasin production continued exponentially, proportionally to cell growth. This study showed that production of tolaasin was not induced until the mid-exponential phase and tolaasin export continued to increase throughout the stationary phase and another study showed the highest rate of toxin production commenced after the stationary growth phase (Murata and Magae, 1996).

3.3.2 Purification of tolaasin

To identify the compound responsible for *P. tolaasii* brown blotch, procedures were designed for toxin isolation (Malcom, 1981, Peng, 1986). The compound responsible for blotch was identified as a polypeptide with molecular weight between 1 and 10 kDa and was first designated as 'tolaasin' (Peng, 1986). The toxin was eventually purified from a cell-free culture filtrate of *P. tolaasii* using a method in which *P. tolaasii* retained full biological activity (Nutkins, *et al.*, 1991). The crude tolaasin preparation was fractionated by HPLC and found to consist of two principal components with molecular weights of 1985 and 1941 that were designated Tol I and Tol II. Tol II was marginally more hydrophobic than Tol I.

To improve the detection of tolaasin and other potential toxins, a *P. tolaasii* strain from *Pleurotus ostreatus* was used to determine optimal parameters for toxin production; such as culture medium, temperature, period of incubation (Shirata, *et al.*, 1995). Using this method, eight different toxins were purified from the culture filtrate, of which toxins '4' and '6' were identified as the same compounds described as Tol I and Tol II respectively (identified in (Nutkins, *et al.*, 1991)). Tox 4 (or Tol I) was found to be the predominant component of the toxins.

In a further study, a *P. tolaasii* strain isolated from *P. ostreatus* was also found that four different toxins with biochemical properties resembling tolaasin (Murata and Magae, 1996). In accordance with their retention time in HPLC analysis, these were named Tox 1, Tox 2, Tox 3 and Tox 4. Tox 3 constituted approximately 94% of the total amount of toxins, whereas Tox 1, Tox 2 and Tox 4 account for approximately 1, 3 and 2% respectively. However, the potential to induce brown blotch of Tox 3 per weight unit is about the same as that of the other toxins. This suggested that the ability to provoke

browning depended on the toxin composition. The chemical structures of the toxins produced by *P. tolaasii* on *Pl. ostreatus* have not yet been determined, however HPLC analyses of these compounds suggests they are related to the toxins comprising tolaasin found in *P. tolaasii* strains mentioned by Nutkins (1991) and Shirata *et al.* (1995).

3.3.3 Structure and comparison of tolaasin to other lipodepsipeptides

Many phytopathogenic fluorescent pseudomonads produce toxins containing peptide moieties similar to the lipodepsipeptide tolaasin that are typically small and are often wholly or partially cyclic. Examples include phaseolotoxin, tabtoxin, tagetitoxin (Stewart, 1971, Moore, *et al.*, 1984, Mitchell and Marshall, 1989). When tolaasin was compared to other described toxins, strains of *Pseudomonas syringae* pv. *syringae* produce the highly similar antimicrobial toxins, syringomycin and syringotoxin (Hutchison and Gross, 1997), syringopeptin (Ballio, *et al.*, 1995) and the syringostatins (Mitchell and Marshall, 1989). Tolaasin (figure 3.1a) clearly has a number of features in common with these latter groups of toxins, including the presence of dehydro and other unusual amino acids. However, tolaasins relatively high molecular weight and the small size of its lactone macrocycle, composed of only five residues, appear to be unique. Tolaasin contains amphiphilic α-helices that are often involved in cellular disruptions via the formation of protein pores in membranes (Mortishire-Smith, *et al.*, 1991). However, in terms of size, the putative helical regions of tolaasin show greater similarity to the central portions of secretory protein signal sequences that to linear helical peptides.

Figure 3-1a structure of tolaasin

3.3.4 Synthesis of tolaasin

The regulation of tolaasin production has been sought by identifying genes involved in pathogenicity, virulence, or the hypersensitive response within the *P. tolaasii* bacterial genome. A study was carried out in which the genetic basis for tolaasin production was sought by screening transposon (Tn*5*phoA) mutants that were unable to produce detectable tolaasin (Rainey, *et al.*, 1993). They found that a gene cluster of 65 kb at one end of a 640 kb *Pac*I chromosomal fragment was required for tolaasin biosynthesis. This chromosomal region was subsequently found to encode three high molecular weight proteins which, when nucleotide sequence coding these proteins was mutated it directly affected the production of functional tolaasin. These three proteins were designated the TL-

cluster and consisted of TL1 (465 kDa), TL2 (440 kDa) and TL3 (435 kDa) (Figure 3-1b) and were thought to be peptide synthetases (Rainey, *et al.*, 1993).

Two other loci essential for tolaasin synthesis were also identified, but their gene products are still not known. One of these is located within the TL-cluster and resides between TL1 and TL2 (defined by the *P. tolaasii* mutant PT182), while an additional gene is located outside the TL-cluster. Small gene(s) may be present upstream of TL3 that may be required for expression of TL3 and TL2. Expression of TL1 may require the presence of functional TL2 and TL3, and similarly, synthesis of TL2 and TL3 may depend upon functional TL1. It is also possible that translational coupling may be involved in the generation of TL1, TL2 and TL3.

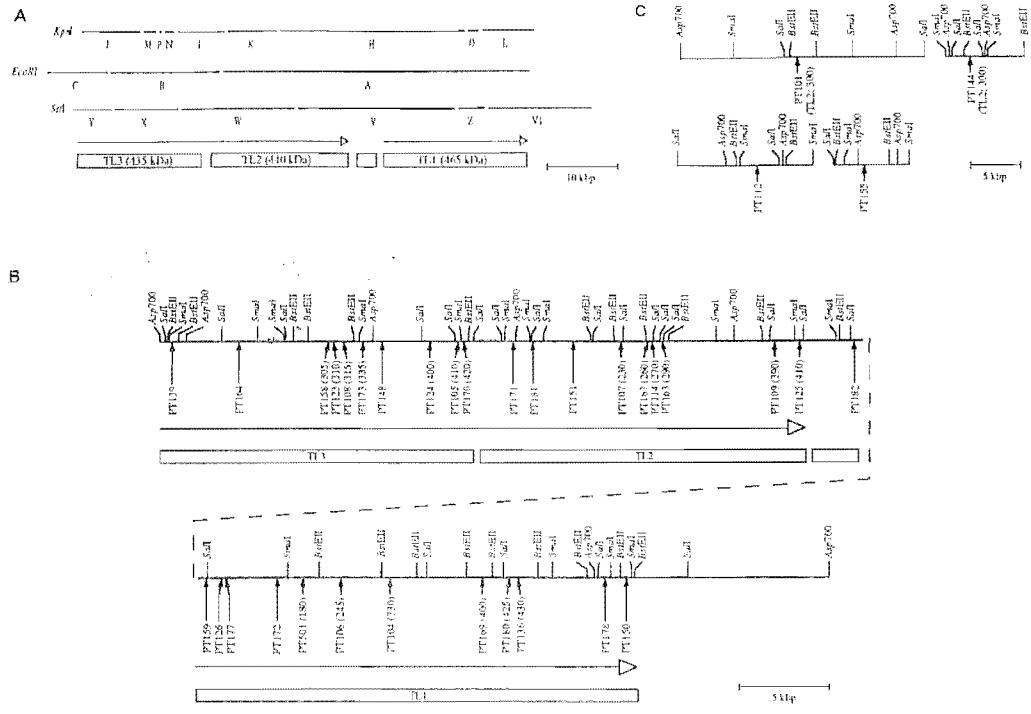

Figure 3-1b Genetic loci involved in *P. tolaasii* tolaasin production (Rainey, *et al.*, 1993). (A) Linkage map of the adjacent *Kpn*I, *Eco*RI and *Sst*I restriction fragments from Southern hybridization analysis of chromosomal DNA isolated from Tn*5* mutants of *P. tolaasii*. (B) Restriction map of the tolaasin gene cluster (TL-cluster) showing proposed genetic organization of TL1, TL2 and TL3. Bracketed numbers indicate the size of the truncated protein where detected and the direction of transcription is indicated by the horizontal arrows. The precise transcription initiation and termination sites are not known and continuity of fragments is indicated by the dotted line. (C) Nearest restriction site maps of DNA flanking Tn*5* in the four mutants where the position and order of restriction sites does not permit placement within the TL-cluster.

TL2 can be transcribed either by read through of TL3 or, albeit weakly, from its own promoter, independently of TL3 expression. This suggests that the 3' end of TL2 and the 5' end of TL3 might overlap, but until the proteins sequences are determined, it is still early to postulate the function. Mutants unable to synthesize tolaasin are unable to cause disease symptoms indicating that a single transposon insertion in a gene is able to prevent the production of both naturally occurring forms of

tolaasin Tol I and Tol II. This demonstrates that Tol I and Tol II are synthesized via the same pathway.

3.3.5 PheN regulation of tolaasin production

Like most virulence factors in bacteria, *P. tolaasii* tolaasin production has been found to be under specific genetic regulation. The *pheN* gene has been reviewed in Section 2.4.2 in respect to its importance in colony transition from 'smooth' to the 'rough' phenotypes. Furthermore, although the *pheN* gene induces the switch from the smooth to the rough forms that results in loss of *P. tolaasii* pathogenicity, the absence of *pheN* gene reduces but does not eliminate pathogenicity. Therefore, the *pheN* gene has been suggested to be a factor that either directly or indirectly affects the expression of the genes involved in tolaasin synthesis (Grewal, *et al.*, 1995). Furthermore, it has also been shown that the production of tolaasin is under regulation by an extra genomic factor that mediates toxin production and efficiency (Mamoun, *et al.*, 1997).

3.3.6 Biological properties of tolaasin

Treatment of mushroom caps with purified tolaasin led to marked disruption of the plasma membrane and vacuole membranes and collapse of the cells walls. This damage was identical to that caused by the inoculation of intact bacterial cultures of *P. tolaasii*. Furthermore, tolaasin has been shown to activate the important browning enzyme, tyrosinase either when extracellularly produced from *P. tolaasii* or in purified form (Soler-Rivas, *et al.*, 1997a). To study the general effects of tolaasin, apart from the wild type of *P. tolaasii*, mutants of *P. tolaasii* were used, such as *P. tolaasii* PT144 that produces a truncated tolaasin (tolaasin-144) that lacks three amino acids from the α-helical region of the molecule (Rainey, *et al.*, 1993). Tolaasin from the wild-type bacteria and tolaasin-144 were both able to induce symptoms when applied on intact mushroom caps (Hutchison and Johnstone, 1993). These effects could be inhibited by addition of $ZnCl_2$[1].

Effect of tolaasin on microorganisms and plants

Tolaasin displays a broad spectrum of activity and is active against a range of organisms including certain bacteria, fungi and plants. Tolaasin was found able to inhibit the growth of numerous fungal cultures (*Agaricus bisporus*, *A. bitorquis*, *Coprinus cinereus*, *Flammulina velutipes*, *Lentinus edodes*, *Pleurotus cornucopiae*, *P. cystidiodies*, *P. ostreatus*, *P. sajor-caju* and *Volvariella bombycina*) by placing disks of cell-free culture filtrates of *P. tolaasii* at the periphery of a mushroom mycelial colony. Similar inhibition was also noticed when bacterial cultures were inoculated on agar alongside the growing mycelial colonies. This observation is relevant to commercial growing as *P. tolaasii* may inhibit the growth of mycelia at the spawn growing stage of mushroom cultivation (Rainey, *et al.*,

[1] The role of Zinc (Zn^{2+}) was found able to inactivate the formation of ion channels in lipid bilayers by tolaasin Brodey *et al.*, (1991)

1991). Purified tolaasin also exerts its activity against some plants. 10 $\mu g.ml^{-1}$ was sufficient to cause cytolysis and necrosis of *Nicotiana tabacum* and *Solanum* sp. leaf cells (Rainey, *et al.*, 1991).

Effect on planar lipid bilayers

The ability of tolaasin to form ion channels in planar lipid bilayers was demonstrated by the observation that adding tolaasin to membranes resulted in movement of ions across the membrane (Brodey, *et al.*, 1991), whereas in this same study purified tolaasin-144 showed no ability to form ion channels in planar lipid bilayers. Other peptides such as melittin and alamethicin have been intensively studied and found to form channels in membranes (Boheim, 1974, Tosteson and Tosteson, 1981).

Effect on erythrocytes – identification of pore formation

Tolaasin was able to induce haemolysis in horse erythrocytes. The rate of lysis was directly proportional to toxin concentration and tolaasin activity was dependent upon erythrocyte concentration (Rainey, *et al.*, 1991). The dependence of lysis upon erythrocyte concentration suggests that lysis (at constant tolaasin concentrations) is reliant upon the rate at which tolaasin molecules insert into, and/or aggregate within, membranes.

Divalent metal ions were effective inhibitors of erythrocyte haemolysis. The most potent inhibitor was Zn^{2+} followed by Mn^{2+}, Co^{2+}, Mg^{2+} and Ca^{2+}. This inhibitory effect of divalent metal ions confirms that the haemolysis results from the formation of pores in the erythrocyte plasma membrane. Divalent metal ions are thought to exert their inhibitory effect by binding to negatively charged groups on the extracellular side of the plasma membrane, near the site of pore formation. The inhibitory effect of Zn^{2+} cannot be due to the prevention of tolaasin binding, but as result of the closure of already formed pores (Rainey, *et al.*, 1991).

Ion channels

The theory that pores formed in the membrane by tolaasin are ion channels is supported by the inability of the altered toxin, tolaasin-144, to form ion channels in lipid bilayers (Brodey, *et al.*, 1991) and to not cause haemolysis of erythrocytes at a concentration at which the tolaasin from the wild-type bacteria is effective (Rainey, *et al.*, 1991).

Biosurfactant properties

Biosurfactants, or surface-active agents, are amphipathic molecules that have both charged and hydrophobic moieties. Alignment of these molecules at liquid interphases results in a reduction in the surface tension at the interphase. Bacteria produce a wide variety of surface-active compounds that are considered to play a major role in the colonization of plants by pseudomonads. Examples of biosurfactants include surfactin, viscosin, and serrawettin (Matsuyama, *et al.*, 1986, Sheppard and Mulligan, 1987, Neu, *et al.*, 1990). These surface-active compounds reduced the surface tension of water demonstrating that tolaasin has biosurfactant properties. Furthermore, tolaasin-144 was also

analysed and its surface-active properties were found to be similar to wild-type tolaasin. As this truncated tolaasin-144 was shown to produce disease symptoms at a concentration similar to that of tolaasin on mushroom caps, and tolaasin-144 was shown not to be influenced by the presence of Zn^{2+}, the surface active properties of tolaasin may be responsible for haemolysis of erythrocytes and may have a key role in causing brown blotch symptoms on mushrooms.

In the case of cut mushroom tissue, the inactivity of tolaasin-144 suggests that ion channel formation is primarily responsible for tissue lysis. However, on the surface of the mushroom cap, similar concentrations of tolaasin and tolaasin-144 cause disease symptoms, which suggests that the biosurfactant properties of these molecules are responsible for the effects. Whether the concentrations of these toxins can become high enough on the mushroom cap for their surface active properties to play a role in disease symptoms is still unknown. It is probable that the concentration of toxin will rise significantly when droplets are placed on the mushroom cap, since the water is rapidly absorbed due to the high internal osmotic pressure of the mycelium (Hutchison and Johnstone, 1993).

Other detergent-like molecules are known to disrupt membrane integrity. Syringomycins and syringopeptins, lipodepsipeptides produced by *Pseudomonas syringae* pv. *syringae*, are known to display potent biosurfactant properties and form ion channel causing necrosis in plants. They provide enough evidence that membrane damage *in vivo* may be due to the detergent-like properties of these molecules (Hutchison and Gross, 1997).

Analysis of the role of tolaasin in colony spread

The rate at which colonies spread across the host's surface may play a significant role in the ability of plant pathogens to colonize their hosts. Molecules such as tolaasin and tolaasin-144 with biosurfactant properties have been speculated to be involved within condensed water on mushroom caps, reducing the surface tension of the drop, and hence the contact angle, will fall. This would allow the water drop to spread, thereby enabling *P. tolaasii* to colonize fresh areas of the mushroom cap (Hutchison and Johnstone, 1993).

3.4 Other browning agents produced by *P. tolaasii*

Recently, compounds other than tolaasin produced by *P. tolaasii* are being studied because of evidence that indicates the presence of other molecules that induce symptoms similar to the brown blotch (Shirata, *et al.*, 1995). These compounds include extracellular enzymes such as proteinases or lipases (Baral, *et al.*, 1995, Baral and Fox, 1997), siderophores to capture the iron required for growth (Munsch, *et al.*, 2000), and exopolysaccharides that mediate the attachment of the bacteria to the fungal mycelium and protect from desiccation.

A study showed *P. tolaasii* can also produce volatile compounds after infecting *Pleurotus ostreatus*. These compounds, named 'tovsins', are different to tolaasin and induced rot on fruitbodies of the *P. ostreatus*. Tovsin caused browning and rotting of mushrooms, inhibited germination of plant

seeds and growth of phytopathogenic fungi, such as *Rosellinia necatrix*, but gave little inhibition of bacterial growth. The quantity of tovsin produced by *P. tolaasii* depended on the mushroom species and its growth media: a large quantity was produced on *P. ostreatus* but little on *Flammulina velutipes*.

A further toxic compound produced by *P. tolaasii* has been identified to produce brown blotch on the mushroom caps and appears to be an aminobenzene with an amylalamine group (Park, *et al.*, 1994).

3.5 Objectives for Chapter 3

This chapter presents the characterization of a fluorescent pseudomonad, designated *Pseudomonas* NZI7 that was isolated from a bacterial survey within a major New Zealand mushroom farm. The hypothesis formulated prior to undertaking of this section of study was the WLA and mushroom cube bioassay combined, provides an efficient screening protocol to identify *Ps tolaasii* isolates. The objectives outlined were:

1 To use the WLA assay to determine the presence of *P. tolaasii* isolates on a selected major mushroom farm.

2 To confirm that WLA+ isolates gave a brown blotch phenotype in mushroom cube bioassay.

From this initial screen an isolate designated NZI7 was found to induces a positive white-line-in-agar (WLA+) assay and produces brown lesions in mushroom tissue bioassay. The subsequent objective was:

3 To use biochemical and genetic analyses to verify that any WLA+ isolates detected are *P. tolaasii*.

Biochemical and genetic examinations demonstrated NZI7 and *P. tolaasii* were dissimilar, therefore:

4 To determine identity of NZI7 by comparison to *P. tolaasii* and selected type species of the genus *Pseudomonas*.

5 To determine degree of similarity of the LDP compound produced by NZI7 with that of *P. tolaasii* tolaasin, and other characterised LDP.

METHODS AND RESULTS

A separate population study from that presented in Chapter 2 was initiated in 1998 to address the presence of *P. tolaasii* on a single major mushroom farm in New Zealand. This farm was experiencing *P. tolaasii* brown blotch symptoms that were significantly reducing marketable yields. Therefore, the presence of *P. tolaasii* from nine locations in and around this mushroom farm was sought in order to determine potential inoculation sites of *P. tolaasii.* Samples in this section of study were not taken from blotched mushrooms as the objective was to attempt to determine inoculation sites of the potential pathogens; therefore sites sampled were a) water reservoirs; b) primary components of mushroom compost; c) primary components of casing layer (peat and limestone); d) compost and casing on mushroom beds; e) walls and floors; f) pickers tools; g) wooden trays and h) soil surrounding the farm. From this survey, 132 pseudomonad isolates were obtained (data not shown) and screened using the WLA assay.

3.6 White line in agar (WLA) assay

From screening all isolated pseudomonads using the WLA assay (as described in Chapter 2.6.5), one isolate was identified from a compost sample obtained from a mushroom bed that produced a white line precipitate (WLA+) in reaction to *P. 'reactans'* NCPPB 1311 (Figure 3-2). This WLA+ isolate was designated NZI7 and was initially assumed to be a *P. tolaasii* isolate based on previous literature reporting the specificity of the WLA assay[1].

3.7 Mushroom tissue bioassay to determine blotch discolouration

Cube mushroom bioassays were carried out (as described in Chapter 2.9) to determine the degree of blotch discolouration caused by NZI7 in comparison to control *P. tolaasii* strains (Figure 3-3). It was observed that NZI7 exhibited equally intense discolouration of mushroom cubes as that produced by inoculation of *P. tolaasii* strains (NCPPB2192T, NCPPB1116, NCPPB741, NCPPB2325, NZ027 and NZ032).

[1] Discussed Section 3.2

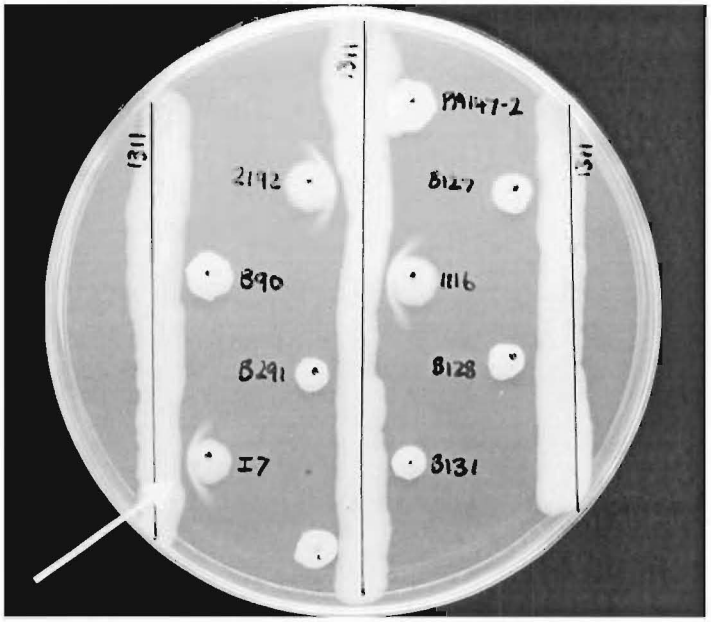

Figure 3-2 Screening *Pseudomonas* strains using the WLA (Wong and Preece, 1979). Vertical inoculations are *P. 'reactans'* NCPPB1311. Lipodepsipeptide precipitation between *P. tolaasii* strains and *P. 'reactans'* NCPPB1311 is observed as a positive 'white line'(indicated by an arrow). 2192, *P. tolaasii* NCPBB2192; 1116, *P. tolaasii* NCPPB1116; provide positive controls; whereas PA147-2, *P. aureofaciens* PA147-2, B128, *P. putida* B128;, *P. fluorescens* B90; provide negative controls. Arrow depicts NZI7 WLA+ with *P. reactans* NCPPB1311.

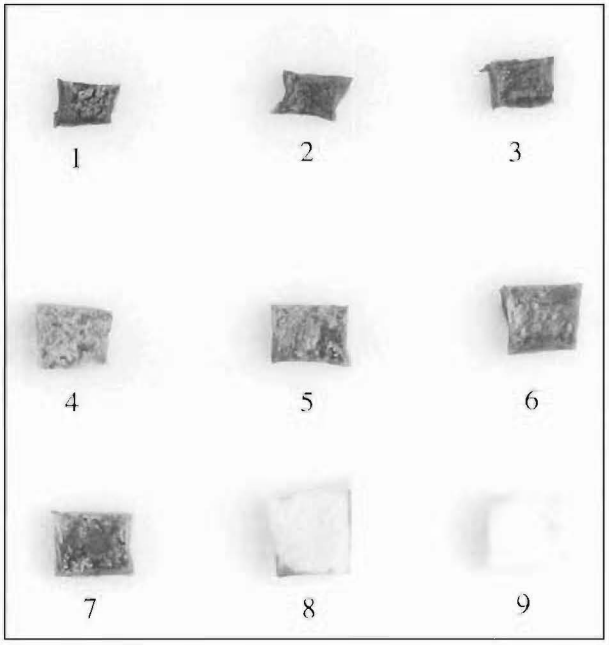

Figure 3-3a Mushroom cube pathogenicity bioassay to determine pathogenic pseudomonads able to induce brown blotch of *A. bisporus* tissue. Positive reaction is indicated by sunken brown lesion with uniform brown discolouration. (1) *P. tolaasii* NCPBB2192^T; (2) *P. tolaasii* NCPPB1116; (3) *P. tolaasii* NCPPB741; (4) *P. tolaasii* NCPPB2325; (5) *P. tolaasii* NZ027; (6) *P. tolaasii* NZ032; (7) NZI7; (8) inoculated with KB; (9) inoculated with ddH_2O. Results depicted are from a 48 incubation at 18-20°C.

3.8 Biochemical and genetic comparisons of NZI7 to *P. tolaasii*

Because both the WLA result and dark discolouration in the mushroom cube bioassay suggested NZI7 to be *P. tolaasii*, it was decided to confirm the identity of NZI7 using biochemical and genetic analyses. For these analyses, NZI7 was compared to *P. tolaasii* strains obtained from international culture collections (Table 3-1).

API 20NE strip analysis

For an initial biochemical analysis, API 20 NE strip analysis was used (as described in Chapter 2.12). Numerical profiles obtained for NZI7 and the *P. tolaasii* strains tested, were compared to the profiles stored in the 1999 Analytical Profile Index Software database (Bio Meriuex). Profiles were different between NZI7 (0152557) and *P. tolaasii* (0156555) (Table 3-1). Based on these profiles, it was determined from the API 20 NE database that NZI7 is a '*P. fluorescens*' isolate (99.4% identity) and likewise, *P. tolaasii* is a '*P. fluorescens*' (99.9% identity). Although both were assigned the same species[1], profile differences suggest they are not the same organism and thus, further clarity was sought using genetic analysis.

Multi locus enzyme electrophoresis (MLEE)

Multi locus enzyme electrophoresis (MLEE) has been used with success in previous studies looking at fluorescent pseudomonad populations within an evolutionary framework (Haubold and Rainey, 1996). MLEE targets 'house-keeping' enzymes. Housekeeping enzymes are critical for cell survival, and therefore genes encoding them are under constant selective pressure to be maintained in the genome. Furthermore, mutations in these genes cannot be significant, as loss of function will result in a non-viable cell and therefore extinction. For these reasons, variation in these genes accumulates very slowly within a given population and mutations are generally selectively neutral. Although only a small number of alleles can be identified within a bacterial population by using this type of variation, high levels of discrimination are achieved by analysing many different loci (Maiden, *et al.*, 1998). MLEE was used in this study to explore the usefulness of applying an established bacterial typing technique to pseudomonads in the mushroom industry.

The MLEE methods used (described in Appendix I(xi)) were derived from previous studies (Selander, *et al.*, 1986, Haubold and Rainey, 1996) for enzymes glucose-6-phosphate isomerase (GPI); glucose-6-phosphate dehydrogenase (G6PDH); malate dehydrogenase NADP (ME); and 6-phosphogluconate dehydrogenase (6PGDH). Scoring of electromorphs was performed as previously described (Selander, *et al.*, 1986), and cluster analysis was carried out using unweighted pair group algorithm averages with the S-Plus statistical analysis package (version 4.5; MathSoft Inc., Seattle). Examination and enumeration of polymorphic variations using MLEE of the enzymes GPI, G6PDH,

[1] The limitations of API 20 NE for species identification of *P. tolaasii* have been discussed in Chapter 2.14.6.

ME, and 6PGDH revealed that NZI7 had different allelic profiles than those exhibited by *P. tolaasii* strains (Table 3-1). Examples of the multiple polymorphic variations observed amongst pseudomonads from this survey are depicted in figure 3-3b.

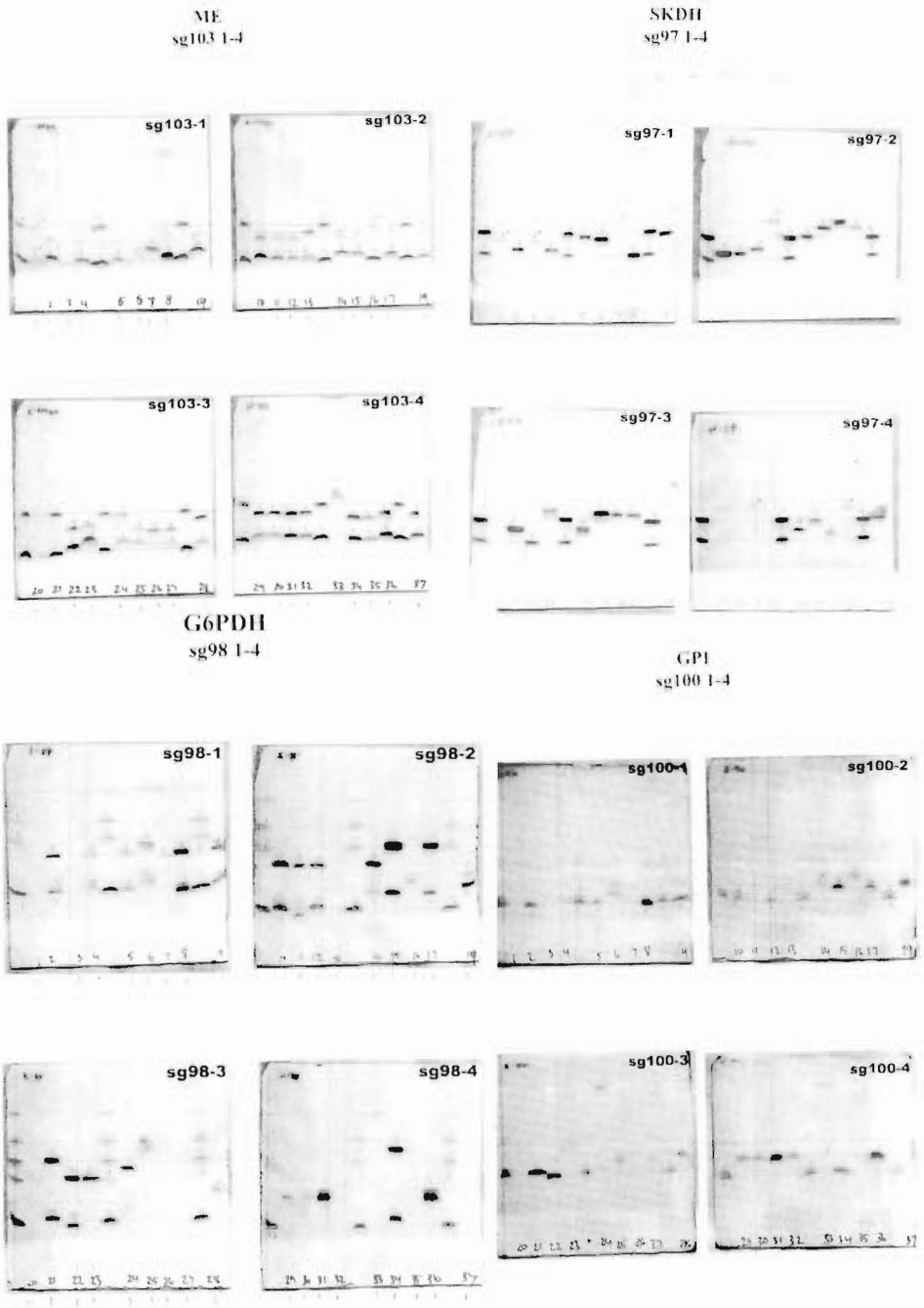

Figure 3-3b Examples of polymorphic variation observed in MLEE cellulose acetate gels for enzymes malate dehydrogenase NADP (ME); shikimate dehydrogenase (SKDH); glucose-6-phosphate dehydrogenase (G6PDH); and glucose-6-phosphate isomerase (GPI) amongst pseudomonads isolated within this survey.

Table 3-1 Bacterial strains used in this section of study and comparative results of API 20NE and MLEE profiles.

Bacterial isolate	Strain designation	API 20NE analysis	MLEE profile[a]	GenBank[b]
Pseudomonas NZI7	NZI7	0152557	3a	AF320993
P. tolaasii	NCPPB 2192T	0156555	1a	AF320988
P. tolaasii	NCPPB 1116	0156555	1a	AF320986
P. tolaasii	NCPPB 741	0156555	1a	AF320992
P. tolaasii	NCPPB2325	0156555	1a	AF320990
P. tolaasii[c]	NZ027	0156555	1a	AF320994
P. tolaasii[c]	NZ032	0156555	1a	AF320995
"P. reactans"	NCPPB 1311	NT	2c	AF320987
P. gingeri	NCPPB 3147T	NT	1d	AF320991

A MLEE profile assigned based on binary data for absence/presence of enzymes in accordance with (Selander, *et al.*, 1986).
B GenBank assignment of 16S rRNA sequences obtained in this study and used for phylogenetic analysis.
C Isolated during Chapter 2.

Repetitive extragenic polymorphic (REP) PCR

REP-PCR has been used in previous studies looking at the homogeneity of *P. tolaasii* isolates (Thorn and Akihiko, 1996) and was successful for the identification of strain differences amongst BCOs in Chapter 2. The primers (REP1R-I and REP2-1 (Appendix VI)) and protocols used for REP-PCR were those described previously in Chapter 2.10 (De Bruijn, 1992). REP-PCR produced indistinguishable banding patterns for *P. tolaasii* strains listed in table 3-1, however NZI7 had a distinctly different profile (Figure 3-4).

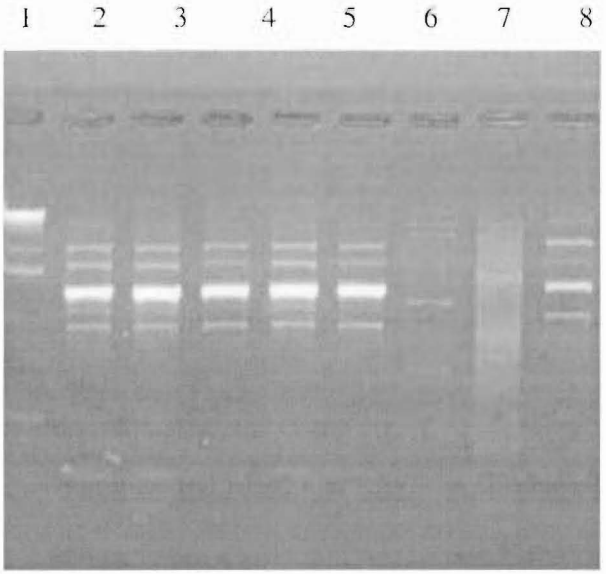

Figure 3-4 0.75% agarose gel showing REP-PCR patterns of *Pseudomonas* strains. Lane (1) 1Kb size marker (LIFE TECHNOLOGIES); (2) *P. tolaasii* NCPPB2192^T; (3) *P. tolaasii* NCPPB1116; (4) *P. tolaasii* NCPPB741; (5) *P. tolaasii* NZ027; (6) *P. tolaasii* NZ032; (7) NZI7; (8) *P. gingeri* NCPPB3147^T; (9) *P. 'reactans'* NCPPB1311

3.8.2 16S rRNA sequencing and phylogenetic analysis

DNA was isolated from pure cultures of bacteria and the 16S rRNA gene was PCR amplified and directly sequenced as previously described in Chapter 2.13. Again, all 16S rRNA genes sequenced in this study were confirmed by determining contiguous overlapping sequences of amplified fragments and the nearly complete 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under the accession numbers listed in Table 3-1.

Comparison of the 16S rRNA amplified from NZI7 was initially sought using BLASTN (version 2.0.14) (Altschul, *et al.*, 1997) to determine similarity of NZI7 to other species and the highest nucleotide identity (97%) was observed to *P. syringae* pv. *savastanoi* (ATCC 13522T). Given this result, 16S rRNA phylogenetic comparisons (described in Chapter 2.13) of NZI7 to validated (ex type) isolates representing the genus *Pseudomonas* (sensu stricto) was performed. Sequences from ex type isolates were retrieved from GenBank (Table 3-2) from a previous study (Moore, *et al.*, 1996).

Table 3-2 Reference sequences (with accession numbers) of 14 validly described pseudomonads (Moore, *et al.*, 1996) used for phylogenetic analyses of NZI7 and *Ps tolaasii* strains.

Bacterial isolate	Strain designation[a]	GenBank Accession
P aeruginosa	LMG1242T	Z76651
P. agarici	LMG 2112T	Z76652
P. asplenii	LMG2137T	Z76655
P. aureofaciens	DSM 6698T	Z76656
P. chlororaphis	LMG 5004T	Z76657
P. cichorii	LMG 2162T	Z76658
P. coronafaciens	LMG 13190T	Z76660
P. ficuserectae	LMG 5694T	Z76661
P. fluorescens biotype A	DSM 50090T	Z76662
P. marginalis pv. *marginalis*	LMG 2210T	Z76663
P. putida biotype A	DSM291T	Z76667
P. syringae pv. *syringae*	LMG1247T	Z76669
P. tolaasii	LMG 2342T	Z76670
P. viridiflava	LMG2352T	Z76671

a DSM Deutsche Sammlung von Mikro-organismen, Gottingen, Germany. LMG Laboratorium voor Microbiologie en Genetica, Rijksuniversiteit, Gent, Belgium. T = Type strain.

Figure 3-5 shows the single shortest neighbor-joining tree based on 16S rRNA gene sequences from 14 selected validly-described *Pseudomonas* spp. (sensu stricto) (Moore, *et al.*, 1996) with NZI7 and *P. tolaasii* isolates.

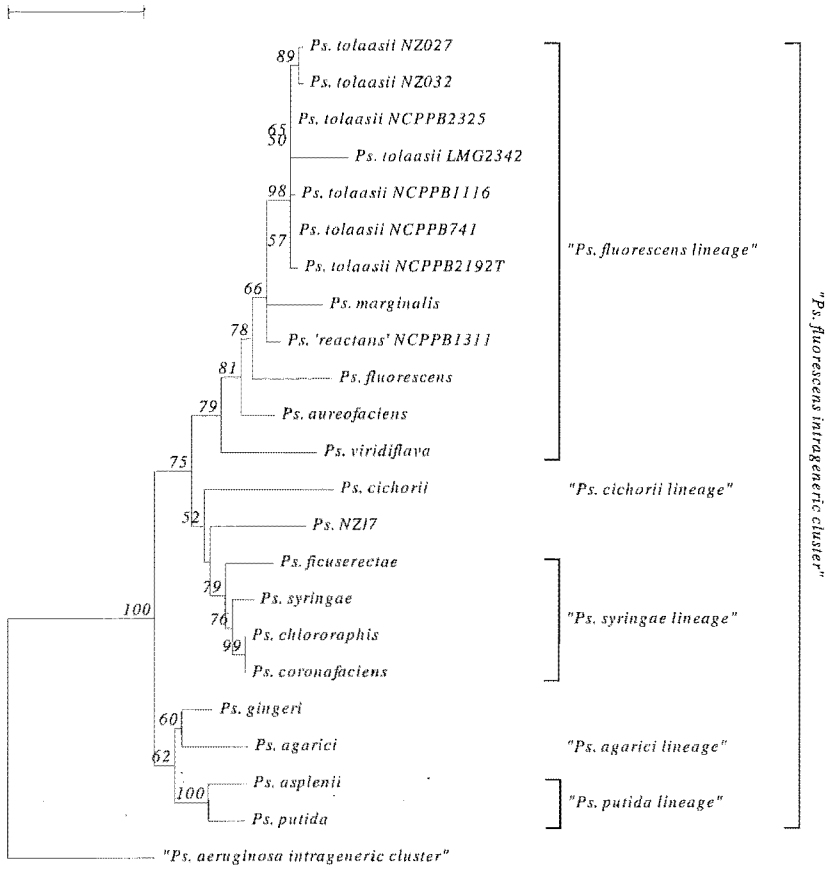

Figure 3-5 The inferred phylogenetic relationships among NZI7 and *P. tolaasii* isolates with other *Pseudomonas* spp. (sensu stricto). Evolutionary distances were determined by pairwise similarities of the 16S rRNA gene sequences and the dendrogram was generated using the neighbor-joining algorithm. Two major intrageneric clusters and five evolutionary lineages are defined as described by (Moore, *et al.*, 1996). Bootstrap proportions of confidence are represented as percentages for those branchings with values greater than 50%.

3.9 Genetic similarity of NZI7 and *P. tolaasii* LDP coding region

Data presented above strongly suggests that NZI7 and *P. tolaasii* are distinct species, however, it was decided to characterise the extracellular compound produced by NZI7 that was reacting in the WLA. Because of the WLA+ reaction, it seemed logical to assume that this compound was similar in chemical and physical structure to that of *P. tolaasii* tolaasin. Therefore, it also seemed plausible that the regulation of the NZI7 WLA+ compound may be similar to that of TL- gene cluster , TL2 etc in *P. tolaasii* (Rainey, *et al.*, 1993). Thus, it was deemed desirable to ascertain the degree of similarity between LDPs from NZI7 and *P. tolaasii*. As the restriction map of the gene cluster for tolaasin biosynthesis has been published (Rainey, *et al.*, 1993), and is suspected to have been sequenced by Prof. Johnstone of Cambridge University, UK (Paul Rainey, pers. comm.), contact with Prof Johnstone was sought. However, Prof. Johnston did not reciprocate communication and therefore it

was determined to directly obtain sequence for the *P. tolaasii* tolaasin biosynthetic cluster within this study.

3.9.1 Sequencing the *P. tolaasii* mutant PT182 Tn*5* insertion site

Paul Rainey kindly provided Tn*5* mutant strains of *P. tolaasii* used to establish the molecular basis of tolaasin production (Rainey, *et al.*, 1993) (Figure 3-1). With these strains, it was determined the most efficient approach would be to identify sequence from the *P. tolaasii* tolaasin Tn cluster and then determine the degree of sequence similarity to that of NZI7. As the tolaasin gene cluster is approximately 65 kb, only a portion of this was deemed practical to sequence (given the scope of this study). From the Tn*5* mutants, *P. tolaasii* PT182 was chosen to obtain sequence. PT182 contains a transposon insertion within a gene (Figure 3-1) that does not affect the expression of TL1, TL2, or TL3, but is nevertheless required for tolaasin synthesis (Rainey, *et al.*, 1993). PT182 was used in this study to determine sequences involved in tolaasin production for several reasons. Firstly, TL1, TL2 and TL3 are all large genes that would take a considerable effort to fully sequence (again given the scope of this study), whereas the genetic region defined by PT182 is smaller. Secondly, sequencing the regions flanking the Tn*5* transposon insertion site of PT182 would allow overlapping sequence to be obtained for both TL1 and TL2, thus providing important information about the arrangement of these genes. Such information would provide superior discriminatory power for NZI7 comparisons to *P. tolaasii.*

To determine the nucleotide sequence flanking the Tn*5* transposon insertion site in *P. tolaasii* PT182 (Rainey, *et al.*, 1993), chromosomal DNA was prepared using the Wizard Genomic DNA Preparation Kit (Promega), digested with restriction endonuclease *Sal*I and ligated[1] to *Sal*I digested pBluescript KS- (pSK-) (Stratagene). Ligation products were used to transform[2] *E. coli* DH5α and plated onto LB medium supplemented with ampicillin (Ap) and kanamycin (Km); (Ap selected for pSK- and Km for Tn*5*). Plasmid DNA from the resulting transformants was prepared[3] and restriction digests showed ca. 300 bp flanking the lac end of Tn*5* and 400 bp flanking the Km end of Tn*5*[4]. Because the insert DNA on either side of Tn*5* was <800bp, direct sequencing of the fragments flanking the Tn*5* transposon was determined using T3 and T7 primers (Appendix VI) that bind the pBluescript KS- vector either side of the *Sal*I site. The T7 primer provided ca. 300bp of nucleotide sequence and oligonucleotide primers were then designed from this sequence to enable determination of whether a similar genetic region existed within NZI7. Oligonucleotide primers pvd1 and pvd2 (Appendix VI) were designed from the DNA sequence flanking the Tn*5* insertion in *P. tolaasii* strain PT182, using the Primer Express computer software (Perkin Elmer).

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[1] Note that restriction digestion and cloning procedures are outlined in Appendix I(vi) and I(vii) respectively.
[2] Transformation (electroporation) is described in Appendix I(viii).
[3] Plasmid DNA preparation is described in Appendix I(iii)c.
[4] Restriction digestion of clones containing Tn*5* is discussed in Chapter 6.3.

3.9.2 LDP gene similarity between NZI7 and *P. tolaasii*

Oligonucleotide primers pvd1 and pvd2 were used to amplify a fragment within the TL cluster from *P. tolaasii* NCPPB2192T and the resulting amplicon was 188 bp and was termed '2192-pvd'. When these primers were used under identical PCR conditions with NZI7, a similar sized (188 bp) amplicon resulted, termed NZI7-pvd. To determine sequence similarity of 2192-pvd and NZI7-pvd, direct nucleotide sequencing of both amplicons using pvd1 and pvd2 was performed. Results showed that NZI7-pvd translated nucleotide sequence shared 97% amino acid identity to translated 2192-pvd (Figure 3-6). The translated NZI7-pvd nucleotide sequence was also compared to amino acid sequences within GenBank and the highest amino acid identity (72%) was observed with the translated product from a syringomycin synthetase (*syrE*) gene from *P. syringae* pv. *syringae* (Guenzi, *et al.*, 1998) (Figure 3-6).

(a)

```
NZI7 pvd      VLSRAYEAPQGPVEVALAQLWQTLLKVEQVGRHDHFFELGGHSLLAVSLVEQMRKQGLD
2192 pvd      VLSRAYEAPEGPVEVALAQLWQTVLKVERVGRHDHFFELGGHSLLAVSLVEQMRKQGLD
```

(b)

```
NZI7 pvd      VLSRAYEAPQGPVEVALAQLWQTLLKVEQVGRHDHFFELGGHSLLAVSLVEQMRKQGLD
P syringae    LISRGYEAPQGEVETLLASIWADVLKVEQVGRHDHFFELGGHSLLAVKLIERMRQVGLS
```

Figure 3-6 Amino acid comparison of translated NZI7-pvd nucleotide sequence with; **(a)** translated *P. tolaasii* 2192-pvd and **(b)** translated *P. syringae* pv. *syringae* syringomycin synthetase (GenBank accession T14593). Shaded regions indicate amino acid identity.

3.9.3 Chromosomal walking to obtain further sequence from *P. tolaasii* 2192

Subsequent to obtaining sequence from the pvd1/pvd2-generated amplicon from *P. tolaasii* 2192T, it was desirable to obtain more sequence to provide further comparison between NZI7 and *P. tolaasii* 2192T. For this purpose, 'directional chromosomal walking' (Mishra, *et al.*, 2002) was undertaken to determine further sequence flanking the PT182 Tn*5* insertion site. The method of chromosomal walking consists of two rounds of PCR: firstly, a primary PCR is performed on genomic DNA using oligonucleotide primers specific to a known sequence in the genome along with four universal 'walker primers' (Figure 3-7-A) that have partial degeneracy (Mishra, *et al.*, 2002); and secondly, the purified product from the first round is used as the template for a second round of PCR using a nested primers (Figure 3-7-C). The walking primers differ from each other at their 3'-end due to degeneracy and each are designed to (theoretically) bind every 256bp in the genomic DNA (Mishra, *et al.*, 2002). At the 5'-end of the primer is a fixed sequence to which the second round nested PCR oligonucleotide can bind. Note that usually four arbitrary primers are used in separate PCR reactions because each

arbitrary primer has a different kinetic likelihood of binding close to the 'known' oligonucleotide primer(s) and therefore, it is likely that at least one of these reactions will generate a PCR amplicon suitable for sequencing.

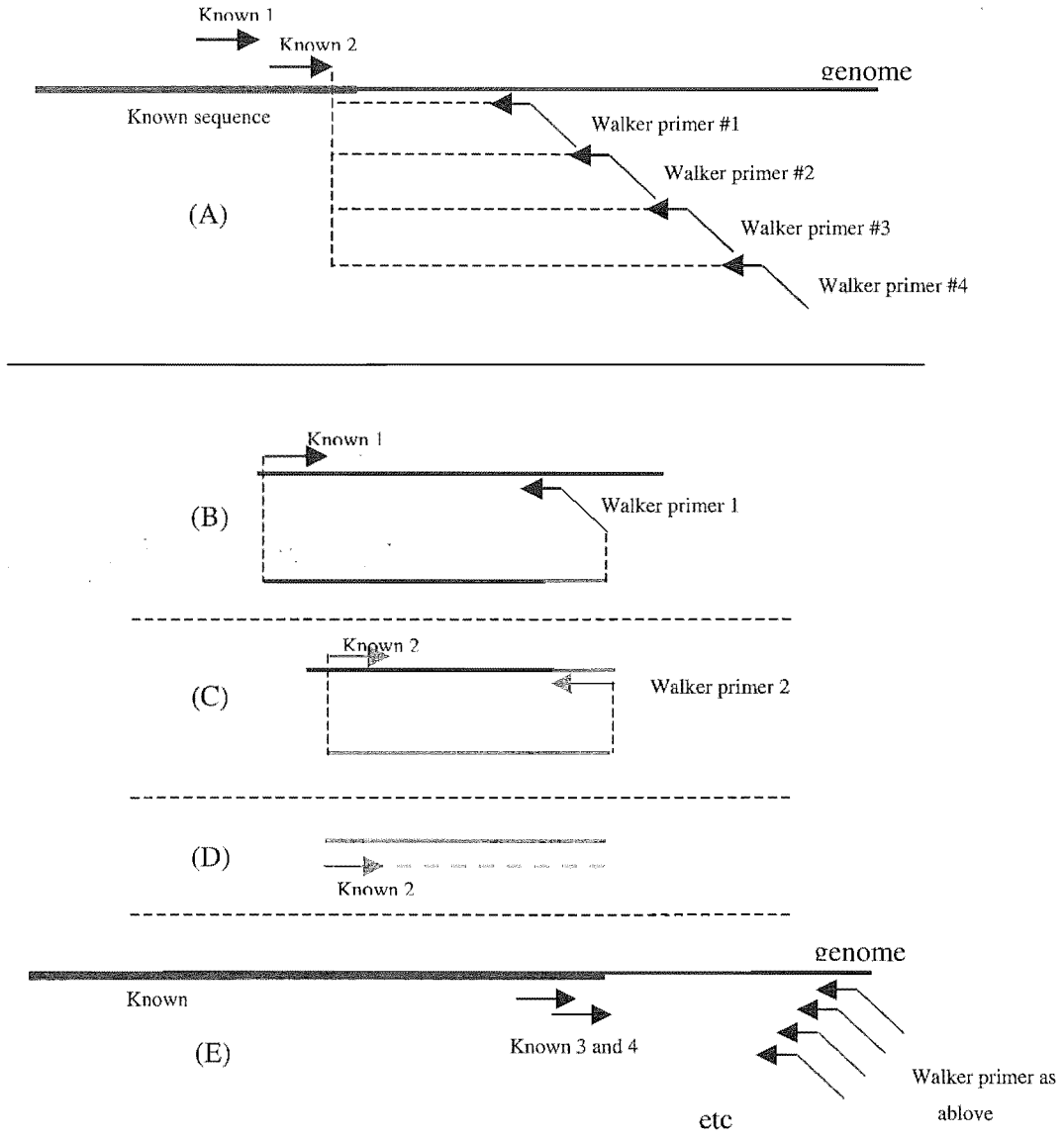

Figure 3-7 General concept of directional chromosomal walking (Mishra, *et al.*, 2002). (A) Using known sequence in the genome, oligonucleotide primers are designed ('Known 1' and 'Known 2'). 'Known 1' is PCR amplified in separate tubes with 'walker primers' 1 to 4 (i.e. 4 separate reactions). (B) One 'walker primer' will generate a PCR amplicon that is used as a template for (C) the second round of PCR in which nested primers ('Known 2' and 'Walker 2' primer) amplify within the first round PCR amplicon. (D) The resulting amplicon can be direct nucleotide sequenced using 'Known 2' primer. (E) Sequence generated is then used to design further Known primers (i.e. 3 and 4) for the next directional chromosomal 'walk'.

Primers were designed to 'walk out' of the pvd1 and pvd2 amplified sequence in *P. tolaasii* 2192 using the following chromosomal walking PCR amplification and sequencing regime (Figure 3-8). Oligonucleotide primers were designed for each round of 'chromosomal walking' and subsequent nucleotide sequencing of second round nested PCR amplicons.).

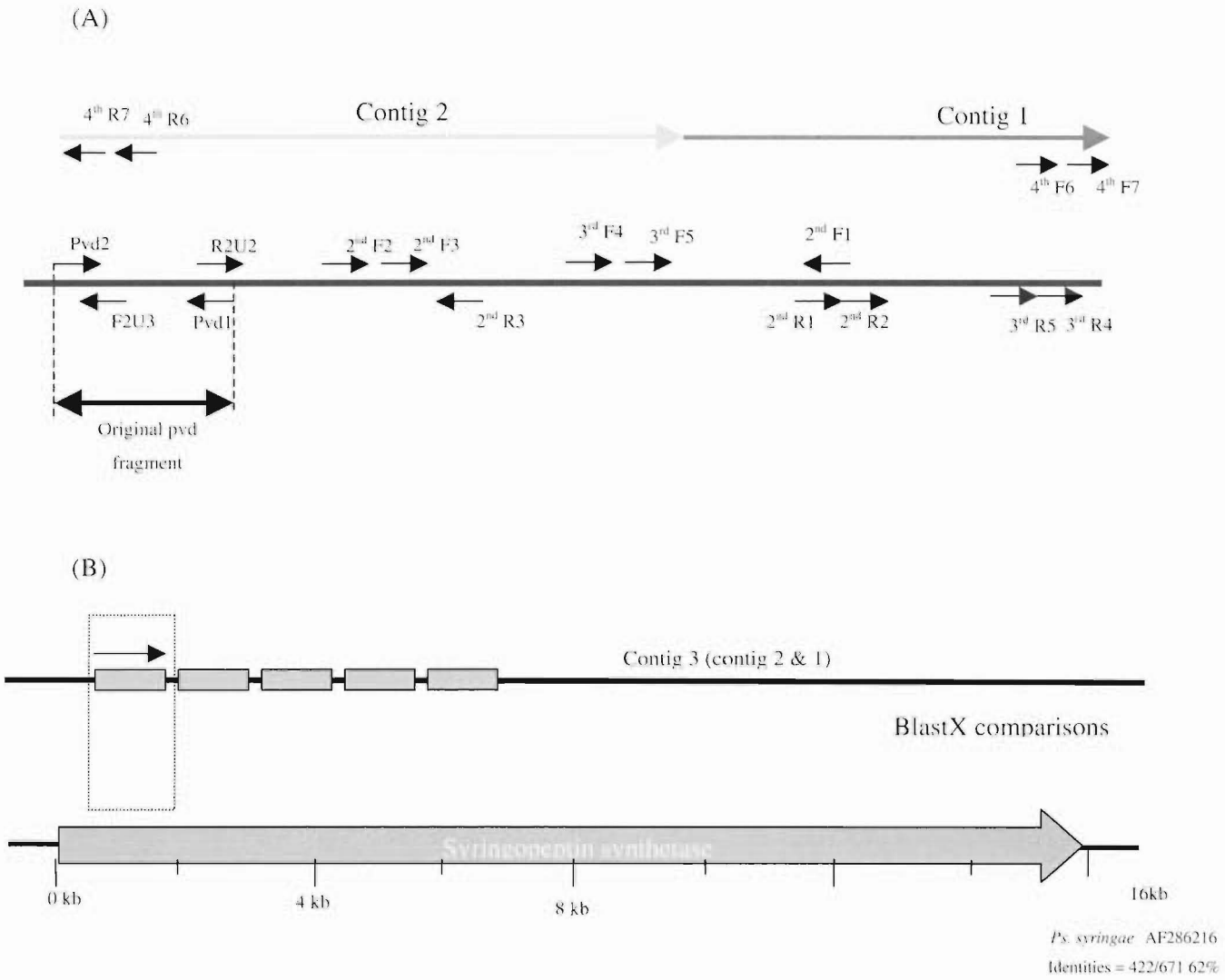

Figure 3-8 (A) Oligonucleotide primers designed to 'chromosomal walk' from the initial PT182 sequence into flanking sequence in *P. tolaasii* 2192T. Two contiguous overlapping sequences were generated, Contig 1 (AY291584) and Contig 2 (AY228241). (B) BlastX comparison to *P. syringae* syringopeptin synthetase (AF286216) showing the five repeat similarity between contig 3 from *P. tolaasii* 2192.

As the method of chromosomal walking requires sequencing of amplicons at each stage, sequence was assembled by contiguous overlapping analysis to generate two contiguous overlapping sequences, contig1 and contig2 as depicted above. The sequence obtained from either direction produced two 'contigs' adjacent to each other. Sequence analysis revealed translation similarity to *P. syringae* syringopeptin synthetase (Accession # AF286216, 57-64% identity) within five repeats (Figure 3-8b).

all exhibiting slight differences in amino acid sequence. Further sequencing of the *P. tolaasii* 2192 LDP cluster was attempted to continue chromosomal walking, however it was found that only non-specific PCR amplifications were obtained. When this region of sequence was further analysed using BlastN and BlastX, it was found that the *P. syringae* syringomycin gene has sequence repeats and oligonucleotide primers were designed within these repeats and therefore generating mismatch nonsense sequence information. No further sequencing attempts were made of *P. tolaasii* 2192 using this method. If it were desired to continue to sequence this region, a suggested approach would be to create a cosmid library to enable subcloning and sequencing of smaller fragments. Oligonucleotide primers generated in Figure 3-8a were used to amplify genomic DNA in NZI7 and that resulted in identically sized amplicons, however, to date, these amplicons have not been sequenced. This further sequence information generated from *P. tolaasii* 2192 has not provided clarity to the NZI7 LDP origin; all the sequence information suggests is that *P. tolaasii* tolaasin and *P. syringae* syringomycin are similar LDPs based on the translated similarity with likely related evolutionary origin. However, this observation would provide a base from which continued study could be developed. An interesting starting point would be a survey of how many other *P. syringae* isolates are able to produce the WLA+ assay given the similarity of LD*P.* Furthermore, given the similarity between tolaasin and syringopeptin

DISCUSSION

P. tolaasii has traditionally been characterized as a fluorescent pseudomonad that causes brown blotch disease of *A. bisporus* and produces a positive WLA result (WLA+) when cultured next to *P. 'reactans'*. The WLA assay was initially used in this study to establish the prevalence of *P. tolaasii* amongst fluorescent pseudomonads from a major New Zealand mushroom farm.

Identification of NZI7

One isolate, designated *Pseudomonas* NZI7, gave a WLA+ result and formed brown lesions in mushroom bioassay consistent with those produced by *P. tolaasii* and was therefore initially assumed to be *P. tolaasii*. However, differences in API NE 20 biochemical profiles suggested NZI7 to be unique. While the carbon assimilation tests included in the API-20NE strip do not effectively discriminate between isolates of *P. tolaasii*, or between *P. tolaasii* and *P. fluorescens* isolates, there were clear profile differences between isolates of *P. tolaasii* and NZI7. This was further supported by genetic analysis (MLEE and REP-PCR), and although other geographically unlinked environmental *P. tolaasii* isolates (NZ028, NZ032) were genetically similar to previously identified *P. tolaasii* isolates, NZI7 was consistently a distinct species.

Phylogenetic analysis of NZI7 based on 16S rRNA

To determine the phylogenetic relationship of NZI7 to *P. tolaasii*, nucleotide sequence analysis of the 16S rRNA gene was performed. The general topology of the tree generated in this study (Figure 3-5) similar to that described by Moore *et al.* (1996) (Figure 2-6) in which the study identified that there are at least two distinct intrageneric divisions within the genus *Pseudomonas* designated: 1) the "*P. aeruginosa* intrageneric cluster"; and 2) the "*P. fluorescens* intrageneric cluster". NZI7 and *P. tolaasii* isolates are observed within the "*P. fluorescens* intrageneric cluster", however, within this cluster NZI7 groups between the *P. syringae* lineage and the *P. cichorii* lineage, which together form a sister clade to the *P. fluorescens* lineage containing the closely clustered *P. tolaasii* isolates.

NZI7 is similar to *P. syringae*

The observed relatedness of NZI7 to the *P. syringae* lineage is not surprising. *P. syringae* has long been considered a genetically diverse species, subclassified into approximately 50 pathovars according to plant pathogenicity (Dye, *et al.*, 1980, Rudolph, 1995) with many strains producing LDP toxins (Segre, *et al.*, 1989, Ballio, *et al.*, 1990, Fukuchi, *et al.*, 1990, Isogai, *et al.*, 1990b, Isogai, *et al.*, 1990a, Ballio, *et al.*, 1991, Fukuchi, *et al.*, 1992, Ballio, *et al.*, 1994, Scaloni, *et al.*, 1997).

Syringomycin and tolaasin similarity

Tolaasin has been shown to have high similarity to the *P. syringae* LDP, syringomycin (Figure 3-6). Syringomycin production is considered to contribute significantly to virulence in *P. syringae* pv. *syringae*. Syringomycin is a necrosis-inducing phytotoxin produced by most strains of *P. syringae* pv. *syringae*, a bacterium that causes infections of parenchymatous tissues of numerous plant species (Mitchell, 1984, Gross and Cody, 1985). Although many strains of *P. syringae* pv. *syringae* exhibit a restricted host range, syringomycin (like tolaasin) is not host specific. Syringomycin disrupts physiological functions within the plasma membrane of host cells (Zhang and Takemoto, 1987), thus eliciting necrosis which resembles at least part of the natural disease syndrome. At low concentrations, syringomycin stimulates a proton pump ATPase in plant membranes, which in turn increases the electrical potential and the pH gradient across the plasmalemma of affected cells (Bidwai and Takemoto, 1987).

Syringomycin resembles many bacterial secondary metabolites in the mode in which its synthesis is regulated (Gross, 1985). Tolaasin clearly has a number of features in common with syringomycin, including the presence of dehydro and other unusual amino acids (Nutkins, *et al.*, 1991). However, tolaasin is unique as it has a high molecular weight and the small size of its lactone macrocycle composed of only five residues. Therefore, although differences exist between tolaasin and syringomycin, they appear to share a common origin.

NZI7 may be a LDP-producing *P. syringae* isolate that has adapted to the mushroom environment niche. Given the similarity between the tolaasin and *P. syringae* produced LDPs, it is likely that syringomycin may produce the same WLA reaction to *P. reactans* and the same disease symptom in *A. bisporus* tissue. However, given the results presented in Chapter 2, the observation of pseudomonads closely related to *P. syringae* (Figure 2-17) causing blotch discolourations is not unexpected, but the WLA+ result of a non- *P. tolaasii* isolate was a novel observation.

NZI7 LDP investigations

As NZI7 exhibited WLA+ result typically associated as a positive identification of *P. tolaasii* tolaasin, genes involved in LDP biosynthesis were compared between NZI7 and *P. tolaasii*. PCR amplification of a DNA coding region between TL1 and TL2 of the *P. tolaasii* tolaasin gene cluster was targeted using PT182 (a *P. tolaasii* Tn*5* generated mutant deficient in tolaasin production (Rainey, *et al.*, 1993)). The region flanking the Tn*5* insertion contains a gene that does not affect the expression of TL1, TL2, or TL3, but is nevertheless required for tolaasin synthesis (Rainey, *et al.*, 1993). The DNA sequences of the resulting 188 bp PCR amplicons from NZI7 (NZI7 pvd) and *P. tolaasii* NCPPB2192T (2192 pvd) were translated and comparison of amino acid composition resulted in strong identity (95.2%) over the entire region (Figure 3-6). However, when NZI7 pvd was compared to the syringomycin synthetase (*syrE*) gene from *P. syringae* pv. *syringae*, identity was significantly lower (72%) (Figure 3-6). Taken together, results from 16S rDNA analysis, MLEE and

REP-PCR suggest that NZI7 is not a *P. tolaasii* isolate, due to its distinctly different genetic characteristics.

Phylogenetic analysis based on the 16S rRNA gene (Figure 3-5) shows NZI7 to cluster most closely to the *P. syringae* lineage. However, the DNA sequence of the NZI7 pvd fragment appears more similar to the gene cluster encoding tolaasin than to that encoding syringomycin. There are at least two possible explanations for this apparently contradictory data. First, NZI7 may be derived from a *P. syringae* isolate that has acquired the tolaasin gene cluster by horizontal transfer among bacterial populations, possibly within a mushroom growing environment. A second possibility is that NZI7 has acquired a mosaic lipodepsipeptide with structural and chemical similarities to both tolaasin and syringomycin, again through horizontal gene acquisition[1]. Studies have revealed that horizontal transfer and recombination of virulence genes plays a major role in generating genetic diversity amongst bacterial species (Kehoe, *et al.*, 1996) and a recent study has suggested that pathovars of *P. syringae* have the ability to acquire at least one gene via horizontal gene transfer (Sawada, *et al.*, 1999). Further examination of the genes involved in LDP production in *P. tolaasii* and NZI7 will be required before further conclusions can be drawn on the origins of the pathogenicity genes in NZI7. It should also be noted that a more detailed investigation of *P. syringae* isolates which produce LDP may identify other LDPs that form a precipitate with WLIP in the WLA given that *P. syringae* is such a diverse species (Palleroni, 1984).

PCR identification of *P. tolaasii* tolaasin

Recently, PCR assays have been published for the detection of *P. tolaasii* (Lee, *et al.*, 2002). These PCR primers were designed to target the genes required for tolaasin production and specificity using nested PCR was reported to be able to detect three *P. tolaasii* cells from 10,000 times as many other bacterial cells. These oligonucleotide primers were not tested on NZI7 and *P. tolaasii* to determine whether these discriminate between the two species. PCR primers directed to *P. tolaasii* tolaasin may also amplify a similar product in non-*P. tolaasii* isolates such as NZI7 and therefore, phenotypic and genetic tests based on LDP production may not satisfactorily assign *P. tolaasii* identity. It is suggested from the results of this study, and that of Chapter 2, that partial 16S rRNA sequencing of the hyper variable region-2 (Moore, *et al.*, 1996) be used to confirm *P. tolaasii* species identity of positive WLA and/or brown blotch pseudomonads.

NZI7 identifies limitation of previous accepted phenotypic assays for *P. tolaasii* identification

It was not the intention of this study to assign species classification to NZI7, but rather report that the identification of *P. tolaasii* based on *A. bisporus* pathogenicity and positive WLA may have limited specificity based on our findings. 16S rRNA analysis in this study provided an efficient method to distinguish and establish relationships between well-resolved *Pseudomonas* species,

[1] The possibility of horizontal transfer in the mushroom environment is discussed in Chapter 8.5.

however, it is acknowledged that the use of 16S rRNA sequences to identify new species is limited without supporting phenotypic and taxonomic experiments (Fox, *et al.*, 1992).

CONCLUSIONS

This section of study reported the isolation and characterisation of a *Pseudomonas* isolate, NZI7, that exhibited all phenotypic criteria described for the identification of *P. tolaasii*, yet showed no genetic similarity. A question that may arise from these findings is that a pseudomonad has been misidentified using phenotypic criteria for *P. tolaasii*, but this pseudomonad still exhibits the disease phenotype of *P. tolaasii* – does this matter? In essence, one could argue that this analysis has still identified an organism that produces brown blotch and therefore, it is still effective in the detection of putative brown blotch organisms producing LPD. However, it is apparent that the WLA is no longer a effective means of positively identifying *P. tolaasii.* Furthermore, results in Chapter 2 showed pseudomonads capable of causing brown blotch symptoms comparable to *P. tolaasii*, however, they had no visible LDP production in the WLA. Therefore, a mushroom cube bioassay that results in a brown discolouration is also insufficient to assign putative species identification of a *P. tolaasii* isolate.

The identification of NZI7 combined with the findings in Chapter 2, further sheds light on the epidemiological complexity of bacterial blotch and highlights the need for continued characterization of bacteria causing disease of cultivated mushrooms. Such studies may yield insights into the genetic origin(s) of *P. tolaasii* pathogenicity factors and identify other brown blotch causing organisms similar to NZI7, as well as their mode of transmission, and the susceptibility of particular mushroom cro*P.*

PRELUDE TO SUBSEQUENT CHAPTERS

Chapters 2 and 3 have identified three major findings: 1) the diversity of pseudomonads capable of causing blotch discolourations of *A. bisporus* is considerably more extensive than previously thought; 2) the organism previously described as *P. gingeri* is not solely responsible for ginger discolourations of *A. bisporus* ('ginger blotch'); 3) a particular blotch discolouration may be caused by more than a single *Pseudomonas* species; and 4) *P. tolaasii* can no longer be efficiently detected by previous phenotypic tests identifying lipodepsipeptide production combined with causing brown blotch on *A. bisporus*. Because these findings show that ginger blotch disease is not caused by a single species, a focused study on ginger blotch could not take place (as was initially the proposal of this thesis prior to commencement). Therefore, a holistic approach was undertaken in an attempt to identify common determinants of blotch amongst the BCOs identified in Chapter 2. For this purpose, the focus of research from this point on was to investigate the BCO/*A. bisporus* interactions using molecular methods.

> *"...The molecular description of microbial pathogenesis reveals a number of common themes that (likewise) involve families of structurally and functionally related adherence factors, toxins, secretion systems and regulators of microbial gene expression. On the other hand, detailed analysis of any specific interactions between pathogen and host uncovers unique mechanisms and molecules. Each pathogen evolves its own particular strategy for manipulation of the host that optimizes microbial survival and transmission..."*

This quote (Manger and Relman, 2000) adequately encompasses the approach laid out in the remaining chapters of this thesis. The presence of these *"common themes"*, (or pathogenicity determinants) were determined for BCOs (Chapter 4) and transposon mutagenesis was used to determine whether these putative pathogenicity determinants are involved in causing *"any specific interactions between pathogen and host"* (Chapters 5 and 6).

Chapter 4

ANALYSIS OF PUTATIVE PATHOGENICITY DETERMINANTS AMONGST BCOs

To prevent a disease, an understanding is required as to how a disease establishes and is expressed. This statement is the foundation upon why extensive worldwide research programs continue to investigate bacterial pathogenicity. Whether bacteria are causative of disease expressed in humans, animals, plants, or fungi, research continues with the aim of elucidating epidemiological and molecular pathogenicity determinants of bacterial pathogens.

It is well understood that environmental conditions are the major factor in the onset and severity of *P. tolaasii* brown-blotch disease (Nair and Bradley, 1980, Van Griensven, 1988). It is also the opinion of this author, as well those expressed by New Zealand mushroom growers (pers. comm.), that environmental conditions play a major factor in the onset and severity of mushrooms with 'ginger blotch' and/or other blotch diseases.

The observation that blotch was formed from 76/95 originally sampled bacterial isolates (80%) in Chapter 2 is suggestive that most pseudomonads are able to form blotch discolourations; given the right environmental conditions (such as those provided in the mushroom cube tissue bioassay). However, because not all of the pseudomonads isolated (20%) caused blotch, it is logical to assume that those BCO isolates that do induce blotch have some physiological characteristic(s) that induce blotch formation[1]. These physiological characteristics are defined as pathogenicity determinants, and these are the focus of research carried out in this chapter.

[1] Although it is not discounted that assay conditions may not have been optimal for blotch discolouration to occur in these 20% of Pseudomonads.

INTRODUCTION AND LITERATURE REVIEW

Bacterial pathogenicity and their virulence factors have been an area of worldwide study for many years with a common aim of elucidating disease processes. Much research has involved the study of pathogens of mammalian cells as medical research is of direct relevance to mankind and thus receives a majority of funding. This literature review has a focus on plant pathogenic bacteria, however, the human opportunistic pathogen, *P. aeruginosa*, is also unavoidably featured heavily as it is arguably the most widely studied and understood of all pathogenic *Pseudomonas* species.

The pathogenicity of a microorganism is defined as its ability to produce disease in a host organism whereas virulence refers to the degree of pathogenicity of a given microbe. Pathogenicity determinants of an organism are any of its genetic or biochemical or structural features that enable it to produce disease in a host. Furthermore, the dynamic relationship between a host and a pathogen is important, since each modifies the activities and functions of the other. The outcome of an infection depends on the virulence of the pathogen and the relative degree of resistance or susceptibility of the host, due mainly to the effectiveness of the host defense mechanisms.

Bacterial pathogenicity genes can be coordinated under major types of global regulatory pathways that allow pathogenic bacteria to adapt to their host environment and cause disease. Pathogenic bacteria are thought to have acquired pathogenicity determinants through evolutionary selection thus enabling them a competitive advantage in their chosen environment (Spiers, *et al.*, 2000). Many bacteria express pathogenicity determinants only in the presence of the host, where the environmental conditions facilitate expression of these genes. Other bacteria produce extracellular compounds constitutively and these compounds may indirectly evoke disease symptoms.

4.1 Determinants of bacterial pathogenicity

Regardless of whether a pathogen of mammalian, plant, or fungal cells, two broad qualities of pathogenic bacteria underlie the means by which they cause disease:

1. The ability to colonise host cells: Mechanisms that facilitate host colonisation include adherence, initial multiplication, ability to bypass or overcome host defense mechanisms, and the production of extracellular substances.
2. The ability to produce toxins: Bacteria produce two types of toxins called exotoxins and endotoxins. Exotoxins are secreted proteins, usually enzymes, from bacterial cells that may either: (i) act directly at the host colonisation site, or (ii) sites removed from the location of bacterial growth. Endotoxins are cell-associated substances that are structural components of cell walls that may induce host response.

4.2 Pathogenicity determinants that promote bacterial colonization

In many plant-associated pathogens, initial stages of disease involve establishing close proximity and bacterial colonisation of the host. Factors that promote bacterial colonization of the host include: contact with the host cell; adhering/colonising the host cell; resisting physical removal; evading host-defense systems; and competing for iron and other nutrients.

4.2.1 The ability to contact host cells

One key element of bacteria persistence is their ability to position themselves in a niche where they can propagate. Numerous positioning mechanisms have been discovered in bacteria that contribute to the virulence of pathogenic bacteria, including: chemotaxis; flagella-mediated motility; and production of pili (Finlay and Falkow, 1997, Ottemann and Miller, 1997).

Flagella motility

Flagella motility provides many different methods of bacterial surface translocation that include twitching, gliding, darting, and sliding (Henrichsen, 1972). Flagella have been speculated in several bacterial functions including: (i) flagella-mediated chemotaxis enables cells to swim towards nutrients associated with a surface; (ii) flagella-mediated motility is used to overcome repulsive forces at a surface; (iii) allowing dividing bacteria to spread along a surface of the host; and (iv) potential involvement of flagella in directly adhering to an abiotic surface.

Pseudomonads are known to be a highly motile genus with specific studies showing the importance of flagella in survival and the initial phase of colonisation (Turnbull, *et al.*, 2001). Pseudomonad flagella have also been studied extensively in respect to their integral role in biofilm formation (O'Toole and Kolter, 1998a) and this is further discussed in following sections pertaining to biofilm formation. Furthermore, *P. tolaasii* have been shown to possess flagella (Masaphy, *et al.*, 1987) and these flagella types have been described as unique in composition from most *Pseudomonas*-flagella (Guilloritrondeau, *et al.*, 1996). These *P. tolaasii* flagella are thought to be involved in positioning of bacterial cells in response to chemical attractants provided by the *A. bisporus* mycelium. The ability to move toward these nutrient sources suggests the hypothesis that *P. tolaasii* has a distinct survival advantage and may also aid colonization of mycelia (Rainey, 1991).

Environmental sensing (chemotaxis)

Sensing various chemicals in the environment and responding to changes is an important environmental adaptation for bacteria in constant interaction with their environment (Alexandre and Zhulin, 2001). Many bacteria possess simple yet effective systems that allow them to regulate numerous cellular functions in response to changes in their surroundings (Stock, *et al.*, 2000). Furthermore, active motility of bacterial cells along chemical gradients is controlled by one of the best-studied signal transduction system; chemotaxis. Chemotaxis is also important in microbial

interactions and colonisation by bacterial cells onto their host (de Wager, *et al.*, 1987) and in particular, bacterial/fungal interactions (Arora, *et al.*, 1983, Lim and Lockwood, 1988). *A. bisporus* mycelia have been shown to produce compounds that can be used by pseudomonads to grow, in particular by *P. putida* (Hayes, *et al.*, 1969). In the water-saturated mushroom cultivation environment, leakage of hyphal contents by *A. bisporus*, and/or production of metabolites is thought to provide a nutrient gradient with ideal conditions for bacterial migration (Samson, *et al.*, 1986). *P. tolaasii* is also a motile bacterium and can modify its position chemotactically towards the mushroom mycelium (Rainey, 1991).

4.2.2 Mechanisms of adhesion (pili and adhesins)

Once a bacterial cell has made contact with the desired host cell, many species have the ability to adhere to the host cell using pili and/or adhesins.

Pili enable some organisms to adhere to receptors on target host cells and thus colonise. Pili (fimbriae) are nonflagellar, filamentous protein appendages originating from the cytoplasmic membrane and are found in virtually all Gram-negative bacteria, but are less common in Gram-positive bacteria. The structure, attachment and genetics of processing, assembly and regulation have previously been reviewed (Hultgren, *et al.*, 1991, Hacker, 1992).

Adhesins are proteins found in the cell wall of various bacteria that bind to specific receptor molecules on the surface of host cells. Adhesins enable the bacterium to adhere intimately to the cell in order to colonize and resist physical removal. Many, if not most bacteria probably use one or more adhesins to colonize host cells (Salyers and Whitt, 2002).

SEM studies of bacteria associated with mushroom mycelia have shown the presence of adhesins facilitating the contact between bacteria-bacteria and bacteria-mycelium. SEM showed some bacteria were attached to hyphae, and to each other, by filament-like structures that were morphologically different from flagella, fimbriae, or pili (Figure 1-8). These structures were observed on washed hyphae in association with attached rod-like bacteria which appeared alone, in pairs, or in aggregates on the hyphal surface (Masaphy, *et al.*, 1987) and appeared to join the bacteria both to the mycelium surface and to each other (Rainey, 1991). Most individual bacterial cells lay flat against the hyphae, with a few attached by one end of the bacterium only. There appeared to be no difference in the arrangement or mode of attachment of the bacterial cells to the hyphae 30 min after washing with distilled water. In all the cases the attached bacteria were apparently joined to the hyphal surface and to each other by the rod-like connections (Preece and Wong, 1982b).

4.2.3 Biofilms

Another form of adhesion to host tissue, is when bacteria produce a capsular polysaccharide matrix (or glycocalyx) to form a biofilm. A biofilm consists of bacterial populations adhering to a surface and may be comprised of either single or multiple bacterial species. In most natural, clinical, and

industrial settings, bacteria are found predominantly in biofilms and not as planktonic (*i.e.* free swimming) cells (Costerton, 1985, Potera, 1996).

Bacterial biofilm formation has been extensively studied (Davey and O'Toole, 2000, O'Toole, *et al.*, 2000b, Watnick and Kolter, 2000) with a focus on understanding the relationship between biofilms and virulence, and it is considered that cellular biofilm adherence is only a prelude to invasion (Finlay and Cossart, 1997). To the knowledge of this author, there have been no descriptions of *P. tolaasii* or other BCOs causing biofilms in current literature.

Biofilm Development

Environmental microbiologists have long recognised that complex bacterial communities existed, however, it wasn't until recently that advances in microscopy and molecular technologies made it possible to closely examine such communities *in situ*. Biofilm formation has been studied with the use of flow and steady state systems enabling the investigation of the structures of biofilms and their physical properties (Lawrence, *et al.*, 1991, Costerton and Lewandowski, 1995, McLean, *et al.*, 1999). Biofilm development initiates when bacteria undergo a transition from a planktonic state to that of firmly attached cells on biotic or abiotic surfaces. Initial attachment is thought to be regulated in part by the nutritional status of the environment (Wimpenny and Colasanti, 1997). After the initial attachment to the substratum, cells are thought to undergo a program of physiological changes that result in a highly structured microbial community. Through growth and active movement across the substratum, spatially distinct clusters of cells, referred to as microcolonies, are formed (Palmer and White, 1997). Although biofilms are highly structured, they are not permanent. Sloughing of cells from the mature biofilm can occur though death, degradation or by cell mediated mechanisms (Palmer and White, 1997, Watnick and Kolter, 2000). This sloughing of cells acts as a means of colonising new surfaces so as to avoid population density mediated starvation of attached bacterial communities (Allison, *et al.*, 1998). Detached cells re-enter a planktonic phase, and the developmental cycle effectively begins again. This is a generalised overview of biofilm formation and structure intended to provide a framework for further discussions (Figure 4-1).

P. aeruginosa biofilm formation

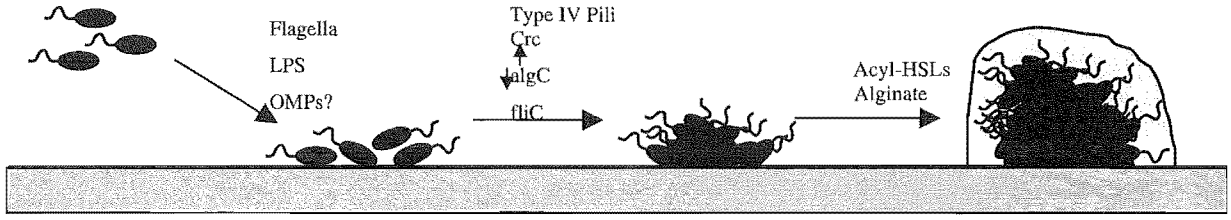

Figure 4-1 Biofilm development in Gram-negative organisms (Davey and O'Toole, 2000). This figure outlines the current model for early stages in biofilm formation in *P. aeruginosa*. (A) Flagella are required to bring the bacterium into proximity with the surface, and LPS mediates early interactions, with an additional possible role for outer membrane proteins (OMPs). Once on the surface in a monolayer, type IV pilus-mediated twitching motility is required for the cells to aggregate into microcolonies. The production of pili is regulated at least in part by nutritional signals via Crc. Documented changes in gene expression at this early stage include upregulation of the alginate biosynthesis genes and downregulation of the flagella synthesis. The production of cell-to-cell signalling molecules (acyl-HSLs) is required for formation of the mature biofilm and alginate may also play a structural role in this process (Davey and O'Toole, 2000).

Biofilm structure

Numerous conditions, such as surface and interface properties, nutrient availability, the composition of the microbial community, and hydrodynamics, can all affect biofilm structure (Davey and O'Toole, 2000). Moreover, it has been shown that biofilms are polymorphic and will structurally adapt to changes in nutrient availability (Stoodley, *et al.*, 1998). Vertical growth of microcolonies and contact with others leads to the formation of connecting channels. In hydrated biofilms, these channels provide high permeability access, allowing nutrients to reach deeply embedded microcolonies as well as the removal of metabolic products that would be harmful in high concentrations (Costerton, *et al.*, 1994). The spatial positioning of bacteria within the biofilm matrix exposes them to varying chemical gradients such as oxygen, pH and iron concentrations (Costerton, *et al.*, 1994, Xu, *et al.*, 1998, Vroom, *et al.*, 1999). In effect, the exopolysaccharide matrix binds the biofilm together, creating a unique microniche for each bacterium within a microcolony.

4.3 Molecular techniques for biofilm studies

In recent years, the advent of advancing molecular methodologies has greatly enhanced the ability to investigate determinants of biofilm formation in different bacterial species (Palmer and Sternberg, 1999). Although studies had shown that transition to a biofilm lifestyle was associated with changes in gene expression and cellular processes, little was known about the types of genes involved in mediating this transition, what extracellular signals are required, and how cellular processes are coordinated to mediate biofilm formation.

4.3.1 Random mutagenesis and abiotic *in vitro* bioassays for biofilm studies

Random mutagenesis approaches for the isolation and characterising of genes involved in biofilm formation have been carried out in several species of bacteria, including; *P. aeruginosa* (O'Toole and Kolter, 1998a), *Escherichia coli* (Pratt and Kolter, 1998), *P. fluorescens* (O'Toole and Kolter, 1998b), *Vibrio cholerae* (Watnick and Kolter, 1999), *Staphylococcus epidermidis* (Heilmann, *et al.*, 1996a) *Streptococcus gordoni* (Loo, *et al.*, 2000) and *P. aureofaciens* (Monds, *et al.*, 2001). Common to all of these studies is the use of a simple *in vitro* screen that allows high throughput assessment of the biofilm properties of random transposon insertional mutants. Biofilm formation in these studies has been operationally defined as the ability to adhere to the surface of PVC/polystyrene microtitre dishes or borosilicate glass tubes. Adhered bacteria are visualised by staining of the cells with either crystal violet or safranin followed by removal of non-attached cells by vigorous rinsing. The inability of a mutant to form biofilms is denoted by its inability to adhere to the abiotic surface.

4.3.2 Biofilms are a result of complex genetic coordination

Transition from initial attachment to development of a mature biofilm requires numerous gene expressions and initiation of regulatory pathways (Kuchma and O'Toole, 2000). Studies using more conventional reporter technology have provided useful information on the changes in gene expression that are associated with biofilm formation. For example, with the use of a *lacZ* fusion, an alginate biosynthetic gene (*algC*) of *P. aeruginosa*, was shown to be upregulated in biofilm cells relative to planktonic cells (Davies, *et al.*, 1993, Davies and Geesey, 1995). Alginate is known to be one of the major components of the exopolysaccharide matrix that encloses *P. aeruginosa* biofilms (Costerton, *et al.*, 1987). These studies gave some of the first indications that contact with a surface can initiate changes in gene expression that direct biofilm development. As an extension to approaches such as these, a library of random transcriptional fusions was generated in *E. coli* K-12, and used to assess changes in gene expression in biofilm cells compared to planktonic cells. (Prigent-Combaret, *et al.*, 1999). Of the fusions generated, 38% were found to be differentially expressed in biofilm enclosed cells. Up-regulated genes were found to be involved in osmoregulation, exopolysaccharide production and cell to cell signaling. These studies are beginning to dissect the specific metabolic differences between biofilm and planktonic lifestyles. The results so far strengthen the view that biofilm enclosed cells represents a distinct physiological state to that of their planktonic counter parts and that two areas are vitally important to biofilm formation; quorum-sensing and adhesions.

4.3.3 Quorum-sensing

Most host-associated bacteria use chemical signals to monitor their population density and control expression of specific genes in response to population density. This type of gene regulation is termed quorum-sensing. Bacteria use quorum-sensing communication circuits to regulate a diverse array of

physiological activities that include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller and Bassler, 2001). Although the nature of the chemical signals, the signal relay mechanisms, and the target genes controlled by bacterial quorum-sensing systems differ, in every case, the ability to communicate with one another allows bacteria to coordinate the gene expression and therefore the behavior of the entire community. Quorum-sensing systems are widespread and have been linked to the regulation of diverse functions in different bacteria; for example, quorum-sensing systems are known to be involved in the regulation of virulence genes in *P. aeruginosa*, conjugal transfer in *Agrobacterium tumefaciens*, swarming motility in *Serratia liquefaciens* and antibiotic production in *Erwinia carotovora* (Reimmann, *et al.*, 1997, Fuqua and Greenberg, 1998).

In general, Gram-negative bacteria have been shown to be able to coordinate their activities through a variety of environmental cues. One such cue are self-produced diffusible molecules that allow cell-to-cell communication within a population, such as the class of signal molecules know as the *N*-acyl-homoserine lactones (AHLs) (Fuqua, *et al.*, 1996, McLean, *et al.*, 1997). AHLs can accumulate in cultures in a cell-density dependent relationship and upon reaching a local threshold concentration, described as a bacterial quorum, AHLs can interact with specific receptors which signal the bacteria to activate differential sets of genes (Salmond, *et al.*, 1995). A detailed study has shown that *P. aeruginosa* has 39 quorum-sensing regulated genes using transposon mutagenesis with promoterless *lac*Z as the reporter (Whiteley, *et al.*, 1999). Furthermore, this study showed many of the identified quorum-sensing-regulated genes were found to code for putative virulence factors or production of secondary metabolites.

4.3.4 Extracellular polysaccharides (EPS) involved in biofilms

Most bacteria are able to produce polysaccharides, either as cell wall polysaccharides (capsules) or as extracellular excretions into the surrounding environment (EPS) (Salyers and Whitt, 2002).

Extracellular polysaccharides involved in biofilm formation

In many cases the stability and vertical development of biofilms have been associated with the production of an extracellular matrix consisting mainly of extracellular polysaccharides (EPS) (Costerton, *et al.*, 1987). These EPS provide one of the most striking examples of an advantage that the biofilm physiological state may provide; biofilm enclosed bacteria can be up to 500 times more resistant to anti-microbial agents (Costerton and Lewandowski, 1995). EPS plays an important role in the composition of the surrounding extrapolymeric substance matrix of bacterial cells in conjunction with proteins, (in some cases) lipids and minor amounts of nucleic acids and other biopolymers (Flemming and Wingender, 2001). The surrounding extrapolymeric substance establishes a microniche in which bacteria are provided with shelter and homeostasis (Davey and O'Toole, 2000)

that is beneficial in sequestering nutrients and providing higher tolerance to biocides (Flemming and Wingender, 2001).

Extracellular polysaccharides produced by mushroom pseudomonads

It has been observed that a variety of acidic EPSs are synthesised by fluorescent pseudomonads associated with mushroom production (Fett, *et al.*, 1995). It is thought that these acidic polymers may protect the bacteria from adverse environmental conditions such as desiccation (Singh, *et al.*, 1992, Osphir and Gutnick, 1994) and may mediate bacterial attachment to fungal mycelia (Rainey, 1991).

4.3.5 Resisting physical removal

Once bacteria have colonised the desired host, it is advantageous for disease formation if the bacterium has the ability to resist physical removal. Such evolutionary adaptations facilitating resistance to removal are those discussed above also used for initial cell-to-cell contact, including the production of pili, cell wall adhesin proteins, and/or biofilm-producing capsules.

4.3.6 The ability to compete for iron and other nutrients

Often the ability of a particular bacterium to express pathogenicity is directly related to the bacterium's ability to compete successfully with host tissue and normal flora for limited nutrients. The more rapid the rate of replication, the more likely an infection may be established. Pathogens, therefore, are able to compete successfully for limited nutrients in their given environment. Generally bacteria compete for nutrients by synthesizing specific transport systems or cell wall components capable of binding limiting substrates and transporting them into the cell. One of the most widely studied examples of this is when bacteria are grown in iron-deficient conditions, they may produce small molecules called siderophores that transport iron Fe (III) into their cells by high-affinity transport systems. The very strong chelating properties of these siderophores may exert an antagonist action against other microorganisms that are no longer able to acquire essential supplies of iron, resulting in growth inhibition. Pyoverdines are the most common siderophores in genus *Pseudomonas* and pyoverdine-mediated iron transport appear to be strictly strain specific (Hohnadel and Meyer, 1988). Three siderophores have been extracted from *P. tolaasii* and identified as chromopeptides that were able to inhibit mycelial growth of *A. bisporus* (Demange, *et al.*, 1990).

4.4 Pathogenicity determinants that elicit response from the host

Many pathogenic bacteria produce pathogenicity determinants that contribute to their virulence by eliciting a response from the host. These can be classified under two major headings of endotoxins (extracellular polysaccharides, or EPSs) and exotoxins (extracellular proteins, or EXPs).

4.4.1 Extracellular polysaccharides (EPS)

All of the various surface components of a bacterial cell are important in its ecology since they mediate the contact of the cell with its environment. The only "senses" that a bacterium has result from its immediate contact with its environment and from these interactions, a bacterium must respond in a way that supports its own existence and survival in that environment. Bacterial surface components may have a primary biological function that has nothing to do with pathogenicity although the bacterial surface may remain an important component in the role of pathogenesis (Schnaitman and Klena, 1993, Meyer, *et al.*, 2001).

Bacterial surface structures may act as: (i) permeability barriers that allow selective passage of nutrients and exclusion of harmful substances (e.g. antimicrobial agents); (ii) adhesins used to attach or adhere to specific surfaces or tissues; (iii) enzymes to mediate specific reactions on the cell surface important in the survival of the organism; (iv) protective structures against host defense systems; and (v) "sensing proteins" that can respond to temperature, osmolarity, salinity, light, oxygen and nutrients resulting in a signal to the genome of the cell.

A Gram-negative bacterial cell wall is relatively thin and is composed of a single layer of peptidoglycan surrounded by the membranous outer membrane and invariably contains a unique component, lipopolysaccharide (LPS or endotoxin), which defines many properties of host-bacterial interactions. Endotoxins are invariably associated with Gram-negative bacteria whether the organisms are pathogens or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, and other leading pathogens (Salyers and Whitt, 2002).

The EPS matrix surrounding the bacterial cell is important in facilitating cell-to-cell contact in biofilm production (discussed previously). EPS also forms a contact barrier between the bacterium/host and it is this matrix that facilitates cell-to-host contact. Often components in the EPS are recognised by the host and will elicit a response, thus EPS can be considered a pathogenicity determinant (Huang, *et al.*, 1993). Furthermore, EPS contain an important class of bacterial extracellular polysaccharides termed galactoglucans that have been observed to be produced by fluorescent pseudomonads (Reed and Costerton, 1987, Fett, *et al.*, 1995). Galactoglucans have not only been proposed to function in biofilm formation, but also to serve as pathogenicity determinants (Liu, *et al.*, 1998).

4.4.2 Extracellular proteins (EXPs)

To aid the invasion of host tissues, bacteria may possess constitutive and inducible hydrolytic enzymes that destroy or derange constituents of host cell membranes, leading to membrane

dysfunction and/or physical disruption (Salyers and Witt, 1994). Since membranes are composed of lipids and proteins, these may constitute a target for attack by such enzymes as proteases and lipases.

Heterotrophic bacteria, in general, produce a wide variety of extracellular enzymes including proteases, lipases, glycohydrolases and nucleases. Although these enzymes presumably have primary functions related to bacterial nutrition or metabolism, they also may facilitate pathogenicity either directly or indirectly. For a protein to be exported into the medium from a Gram-negative bacterium, it must be translocated through both the inner and outer membranes. Most exported proteins use the general transport system and have a signal sequence to get them though the inner membrane, but they must use special systems to get them through the outer membrane. Most of the members of the genus *Pseudomonas* produce very active extracellular enzymes, in particular proteinases and lipases which cause spoilage in protein-rich substrates such as is found in the milk industry and mushroom sporophores (Morihara and Homma, 1985, Carnicero, *et al.*, 1990).

4.4.3 Proteases

Proteases are a major class of enzymes that execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell, whereas intracellular proteases play a critical role in the regulation of metabolism.

Moreover, proteases occupy a pivotal position with respect to commercial fields in which much research has focused for either: a) the overproduction of protease enzymes for various commercial applications in the food, detergent and pharmaceutical industries; or b) if virulence of a particular bacteria is related to the secretion of extracellular proteases, these proteases are studied to understand the basis of their pathogenicity and to develop therapeutics against them. Through commercial enterprise, much knowledge of proteases has been gained.

P. tolaasii proteases

P. tolaasii was found to produce an extracellular metalloprotease very similar in most respects to those secreted by other *Pseudomonas* species (Fairbairn and Law, 1986). The protease was produced mainly during the exponential phase of growth and increased slightly during the stationary phase. The effect of this protease in the infection of the mushroom sporophores is still unknown but it may facilitate the damage caused in the mushroom (Baral, *et al.*, 1995).

4.4.4 Lipases

Lipases are shown to facilitate the infection by disruption of the host membranes. Lipases from many pseudomonads form aggregates of various size (Stepaniak, *et al.*, 1982). Many Gram-negative bacteria produce and secrete lipases into the extracellular medium. Examples studied include *P.*

aeruginosa (Wohlfarth, *et al.*, 1992), *P. alcaligenes* (Gerritse, *et al.*, 1998), *Burkholderia glumae* (Frenken, *et al.*, 1992) and *B. cepacia* (Jorgensen, *et al.*, 1991).

P. tolaasii lipases

Another extracellularly produced bacterial enzyme that could be involved in the infection process of *P. tolaasii* is lipase. Extracellular lipases were first shown in *P. tolaasii* by 'lipolysis of margarine' (Lelliott, *et al.*, 1966) and lipases have been further described by Wong, *et al.*, (1982). Again the role of this lipase in the infection of mushroom was not deduced, however, the authors suggested it requests a further study (Baral and Fox, 1997).

Phospholipases

As phospholipids and proteins represent the major chemical constituents of the host cell envelope, enzymes capable of degrading these, *i.e.* phospholipases, are most likely to be involved in membrane disruption and therefore pathogenicity. Phospholipases are considered virulence factors for bacterial species that cause dissimilar disease syndromes, ranging from infections causing massive tissue destruction such as gas gangrene to food borne listeriosis (Titball, 1993, Songer, 1997). The best-characterised phospholipases are phospholipases C (PLC) that hydrolyse phospholipids to release the phospho-head group and diacylglycerol (DAG). Many PLC are hemolytic and are thought to directly cause tissue destruction, destabilizing host cellular membranes by hydrolyzing membrane phospholipids. In addition, bacterial PLC have the potential to exert profound effects on the host indirectly by the production of lipid second messengers that modulate host cell signaling pathways. Furthermore, many species of pathogenic bacteria (*Clostridium perfringens*, *Listeria monocytogenes*, *P. aeruginosa*, *Bacillus cereus*, *Rickettsia* and *Corynebacterium pseudotuberculosis*) and pathogenic fungi have been identified as having phospholipases that play a critical role in pathogenesis (Ghannoum, 2000).

4.4.5 Chitinases

Chitin is a homopolymer of β-1,4-*N*-acetyl-D-glucosamine (GlcNAc). Chitin is one of the most abundant natural polymers present within many structures, including the cell walls of many fungi and algae, exoskeletons of insects, nematodes and shells of crustaceans. Selected species of the genera *Serratia, Bacillus*, *Vibrio* and *Pseudomonas* are reported to be able to secrete several chitinolytic enzymes and chitin-binding proteins (Watanabe, *et al.*, 1990, Bassler, *et al.*, 1991, Watanabe, *et al.*, 1997, Folders, *et al.*, 2001, Vogan, *et al.*, 2002). The production of chitinases and chitin-binding proteins is often substrate regulated with synthesis repression occurring in rich medium, and synthesis induction when strains are grown in minimal medium supplemented with chitin (Keyhani and Roseman, 1999).

The mushroom soft rot bacterium *P. gladioli* pv. *agaricicola* was observed to cause pitting when inoculated onto tissues of several commercially important Japanese cultivated mushrooms (Gill,

1994). Scanning electron microscope studies demonstrated the sequential removal of hyphal wall layers, thereby exposing the chitin skeletal matrix, which in turn was degraded. Culture plate assays revealed that *P. gladioli* pv. *agaricicola* produces chitinase and this, coupled with earlier evidence of a beta-glucanase enzyme, accounted for the degradative ability of the pathogen (Gill and Tsuneda, 1997). Chitinase activity of mushroom tissue is associated with severe tissue degradation (Gill and Tsuneda, 1997), symptoms of which have not been associated with *P. tolaasii* or other published BCOs.

4.4.6 Extra-genomic factors (*i.e.* plasmids encoding virulence factors)

In addition to the bacterial chromosome, bacteria may contain one or more small, circular macromolecules of DNA known as plasmids. Plasmids usually contain no more than 1-5% of the DNA found in the bacterial chromosome. Plasmids do not normally contain the genetic information for the essential metabolic activity of the bacterial cell, but rather, genetic information for a phenotype(s) that may give its hosts a selective advantage within a given niche. Examples include resistance to antibiotics, tolerance environmental toxicity (e.g. heavy metals) and production of virulence genes required for bacterial pathogenicity (Gulig, *et al.*, 1993).

Plasmids are ubiquitous in a wide variety of bacteria isolated from diverse environments (Salyers and Whitt, 2002). Some plasmids mediate their own transfer from donor to recipient cells by conjugation that requires cell-to-cell contact. These conjugative plasmids carry transfer (tra) genes that code for functions required for bacterial conjugation (van Elsas, *et al.*, 1988). Plasmid replication is carried out by same enzymes that replicate the chromosome of the host cell, and they are distributed to daughter cells along with the host chromosome during cell division. Transmission of plasmids is a mechanism by which specialised information can be transferred from one bacterium to another.

Ralstonia (*Pseudomonas*) *solanacearum* was shown to contain the plasmid pJTPS1 that mediates a shift between pathogenic and nonpathogenic colony states (Nigishi, *et al.*, 1993). This study prompted an investigation into *P. tolaasii* strains and results revealed a similar extra-genomic factor was involved in the mediation of tolaasin production and caused reduced pathogenicity, but not total non-pathogenicity (Mamoun, *et al.*, 1997).

4.5 RATIONAL OF THIS SECTION OF RESEARCH

When reviewing literature concerning previously reported pseudomonads that cause blotch disease, the prominent organism for comparison was *P. tolaasii.* As discussed in previous chapters, the primary causal mechanism of *P. tolaasii* blotch is the production of the lipodepsipeptide, tolaasin. Although other studies have also shown pseudomonads associated with mushrooms to also produce lipodepsipeptides (such as *P. reactans* (Mortishire-Smith, *et al.*, 1991) and *Pseudomonas* NZI7 (Chapter 3)), lipodepsipeptide production was only observed amongst selected BCOs (Table 2-2) and could not be consistently correlated with any one given blotch discolouration. Therefore, it appeared that the majority of BCOs were inducing blotch discolourations by mechanisms other than that of lipodepsipeptide production. Without prior knowledge of pathogenicity determinants amongst the BCOs, this section of study was considered exploratory research in which the 33 BCOs isolated in Chapter 2 were assessed for selected pathogenicity determinants identified in other studies.

4.6 Objectives for Chapter 4

With the hypothesis that a common pathogenicity determinant(s) that induces blotch discolouration of *A. bisporus* is present amongst BCOs, this section of research set out to address the following objectives:

1. To design assays that efficiently analyse BCOs for their ability to exhibit putative pathogenicity determinants (PPDs): namely biofilm formation, motility, auxotrophy, protease, lipase, chitinase, and siderophore production.
2. To select a single BCO amongst the 33 previously described pseudomonads (Chapter 2) representative of having PPDs in Objective 1 for a focused transposon mutagenesis study.

METHODS AND RESULTS

4.7 Determination of pathogenicity determinants amongst BCOs

It was initially deemed desirable to identify common pathogenicity determinants amongst the 33 BCOs identified in Chapter 2 that may account for the universal ability to cause blotch discolouration of *A. bisporus* tissue. The following putative pathogenicity determinants (PPDs) were chosen for assessment and appropriate assays for determination of these PPDs were developed, namely biofilm formation, auxotrophy, siderophore, protease, chitinase and lipase production.

4.7.1 Development of an abiotic biofilm assay for BCOs

An *in vitro* biofilm assay based on the method described previously (O'Toole and Kolter, 1998b) was developed for use with BCOs. The main parameters of this assay are determining an appropriate surface for attachment, choice of growth media, and optimal time of incubation. BCOs were assessed for their ability to form biofilms in conditions in which these three parameters were varied. This analysis served as a means of defining the most appropriate assay conditions for the upcoming mutagenesis screen.

Growth media

Two main types of media (media constituents are listed in Appendix II) were assessed for their capacity to support biofilm formation by BCOs at an incubation temperature of 28°C; M9 minimal media supplemented with glucose (M9) and Luria Bertani (LB) broth. Varying conjointly with media were incubation times (varied from 10 to 24 hrs). Initially, PVC was used as the surface for attachment. Results showed that biofilm formation was observed when BCOs were grown in either M9, or LB, or both. (Table 4-1). That is, some isolates that did not form biofilms in LB, formed biofilms in M9 whereas some BCOs were able to form biofilms in both media. For example, isolates NZ006 and NZ065 formed a biofilm in M9 but not in LB, whereas, isolates NZ007, NZ009, NZ011 and NZ096 were deficient in biofilm formation in M9 but able to produce a biofilm in LB.

When LB was made up at the recommended concentration of constituents (Appendix II) it was observed that a high level of background staining resulted (see Appendix I(xii)c for staining procedures) that is, the negative control was showing staining within the well also. It was presumed this background staining was therefore due to the high concentration of nutrients being present in the LB media. In an effort to eliminate this problem, BCOs were tested for their ability to form biofilms in various dilutions of LB media. It was found that a 1:10 dilution of LB media was the lowest dilution that would support strong biofilm formation as well as reduce background staining to

negligible levels. This medium was designated L10 and unless otherwise stated, was used for all biofilm assays involving BCOs and their derivatives.

Analysis of biofilm formation by the 33 BCOs grown in L10

Further to the above assays, all 33 BCO isolates were re-analysed for the ability to form abiotic biofilms in PVC microtitre plates using L10 instead of LB (full concentration) as the growth medium. It was found that the results did not alter, but the biofilm was more easily observed with the background staining at a minimum. 22/33 BCOs formed strong biofilms, 7/33 formed weak biofilms (NZ009, NZ060, NZ066, NZ096, NZ099, NZ111, and NZ113) and 4/33 did not form visible biofilms using these abiotic conditions (NZ006, NZ064, NZ065, and NZ081) (Figure 4-2). Also tested were culture collection isolates previously described as causing blotch and it was shown that *P. tolaasii* strains (NCPPB 2192, NCPPB 1116, NCPPB2325) showed strong biofilm production, whereas *P. gingeri* NCPPB 3147, *P. gingeri* NCPPB3146 and *P. reactans* NCPPB 1311 produced weak biofilms (Figure 4-2).

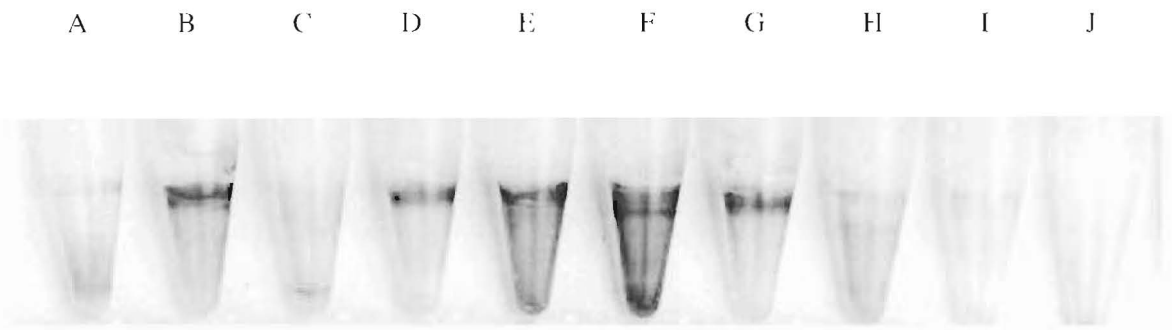

Figure 4-2 BCO isolates from Chapter 2 were analysed for biofilm formation in L10 media on PVC microtitre dish assays. Depicted are: a very strong biofilm formation on the whole tube (E) NZ014 and (F) NZ092; strong biofilm production at the media/oxygen interface (B) NZ052, (D) NZ103 and (G) *P. tolaasii* 2192; weak biofilm (A) NZ060, and (H) *P. gingeri* 3147 and (I) *P. reactans* 1311; and negative (C) NZ006 and (J) uninoculated L10.

It should be noted here that positive biofilm formation was easily viewed, but marginal biofilms described as 'weak' were often a subjective decision based on (at least) three separate assays in comparison to negative controls.

Growth on varying abiotic surfaces

BCOs were further screened for their ability to form biofilms on polypropylene and borosilicate glass surfaces when grown in L10. Again, observations were made that biofilms were not produced by some BCOs on some varying types of microtitre plates, whereas they were on other surfaces (Table 4-1). PVC was chosen as the surface for subsequent biofilm assays due to it being the most consistent

surface that BCO biofilms were formed and its availability, and low cost made it ideal given that numerous assays were to be performed with the mutagenesis screen in the following chapter.

Table 4-1 Comparative biofilm results of BCOs in L10 and M9 media on microtitre plates made from different constituents. (– no growth, + biofilm present, ++ strong biofilm). Note that results were the same between initial assays using LB (full concentration) and L10

Designation	PVC	PVC	Polypropylene	Glass
	M9	L10	L10	L10
NZ 006	+	-	+	++
NZ 007	-	++	+	-
NZ 009	-	+	+	-
NZ 011	-	++	+	-
NZ 014	++	++	++	++
NZ 017	+	++	++	-
NZ 024	++	++	++	++
NZ 027	+	++	-	-
NZ 031	+	++	-	++
NZ 032	+	++	-	-
NZ 039	+	++	+	++
NZ 043	++	++	+	++
NZ 047	++	++	+	++
NZ 052	++	++	++	-
NZ 059	++	++	-	-
NZ 060	+	+	-	-
NZ 062	++	++	+	++
NZ 064	+	-	-	+
NZ 065	+	-	++	-
NZ 066	++	+	-	-
NZ 081	-	-	+	-
NZ 092	++	++	+	+++
NZ 096	-	+	-	++
NZ 097	++	++	++	++
NZ 099	+	+	-	-
NZ 101	++	++	+	++
NZ 102	+	++	+	+
NZ 103	+	++	+	+
NZ 104	+	++	+	+
NZ 111	+	+	-	+
NZ 112	++	++	+	-
NZ 113	+	+	-	-
NZ 124	++	++	+	+
P. tolaasii 2192T	+	++	+	-
P. reactans 1311	-	+	-	-
P. gingeri 3147T	-	+	-	-

Time parameters

Experiments analysing BCO biofilm formation in L10 over time showed that inoculation of assay wells with a 1:50 dilution of a saturated bacterial culture resulted in reproducible detection of biofilms after 12 hrs growth at 30°C. However, strong biofilms were seen anywhere between 8 to 18 hours

growth (Figure 4-3). Interestingly, biofilms were also seen to show signs of degradation when incubated for times in excess of 24-26 hours. Degradation refers to the visually detectable breaking up of uniform ring patterns at the oxygen/media interface.

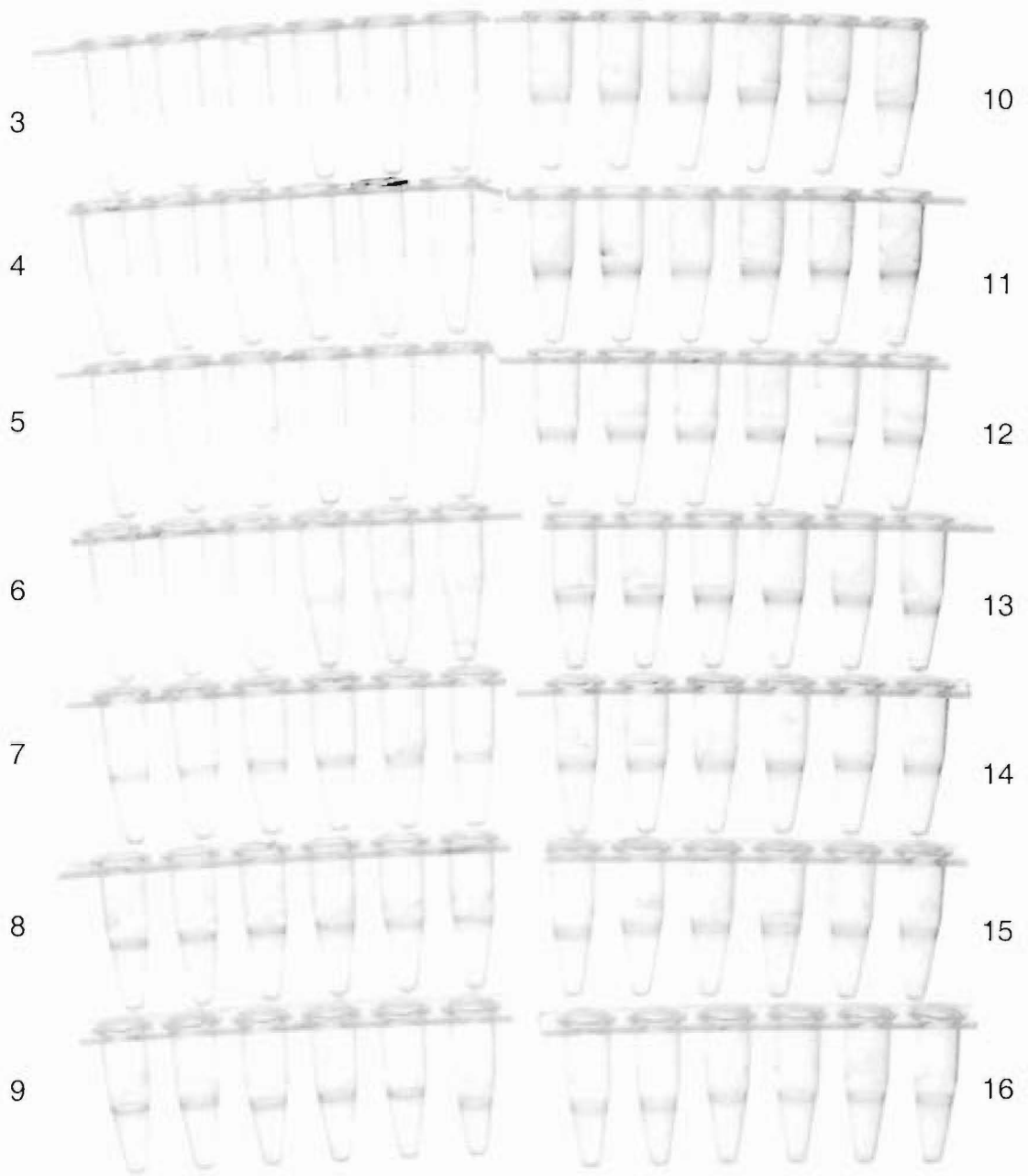

Figure 4-3 Biofilm formation of a selected BCO (NZ103) on PVC, after incubation at 30°C at 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 hours.

Summary of optimal biofilm conditions

In summary, the standard conditions for the assessment of biofilm formation by BCOs were 12 hours growth at 30°C in L10 inoculated with a 1:50 dilution from a stationary phase culture. In the context of this assay, a biofilm is operationally defined as the adherence of bacteria to PVC at the oxygen media interface, as visualised by crystal violet staining (Appendix I(xii)c).

4.7.2 Flagella mediated motility assays

Motility has been well defined as a conditional requirement of pathogenicity as a means of a bacterium becoming in close proximity to its host through such adherence processes as chemotaxis. Furthermore, motility has also been deemed necessary for bacterial biofilm formation. (de Wager, *et al.*, 1987, Lawrence, *et al.*, 1987, Fletcher, 1988, DeFlaun, *et al.*, 1994, Korber, *et al.*, 1994). Due to these observations, all BCOs were characterised in terms of their flagella mediated motility phenotype using motility agar.

Motility agar analysis

Motility agar analysis of BCOs consisted of using 0.3 % 'water agar' plates (Appendix II). For the purpose of this assay, motility was defined as the ability of bacteria to actively swim away from the point of inoculation[1]. Characteristically, functional flagella mediated motility is represented by uniform movement in any one direction from the point of inoculation. As such a symmetrical halo of bacteria is formed. Non-motile bacteria, remain static at the point of inoculation and flagella motility impaired bacteria have also been shown to form irregular halo production indicating disruption in genetic regulation of flagella synthesis. Of the 33 BCO candidates, 33 were observed to have symmetrical halos when grown on motility agar suggesting functional motility.

4.7.3 Auxotrophy

As environmental fitness of a given organism greatly enhances its ability to colonise and become pathogenic, all BCOs were screened for auxotrophy by assessing their ability to grow on M9 minimal media (M9, Appendix II). Of the 33 BCOs, all were found to be auxotrophic showing strong growth characteristics under these conditions.

4.7.4 Siderophore production

Fluorescent siderophore production was assayed qualitatively by growing BCOs on the iron limiting media, Kings medium 'B' and PMM (Appendix II). Siderophores are compounds that are readily visualised in both natural light and under UV light (254 nm) and under these conditions

[1] Figure 5-4 shows a representative positive motility phenotype (NZ103 WT).

fluorescence is assumed to be siderophore production. All BCOs exhibited fluorescence comparable to each other and the control strain *P. fluorescens* SBW25 (Rainey, *et al.*, 1994).

4.7.5 Extracellular protease production

Extracellular protease production was assayed qualitatively by growing mutants on media containing non-fat milk powder (Milk Agar, Appendix II). If extracellular proteases are produced, they catalyze hydrolysis of the large protein component, casein, in the milk powder (the component that makes milk appear white). As milk powder produces a white agar plate, any proteolytic activity is visualized by a clear halo formation. The halo is semi-quantitative in that it can be measured to estimate the amount of protease production from any given colony relative to time. As well as being an easy phenotype to characterise, as discussed in the introduction, production of proteases has been identified as a potential pathogenicity factor in *P. tolaasii* (Baral, *et al.*, 1995) and is also well recognized in their contribution to the pathogenicity of many other bacterial pathogens. Of the 33 BCOs 10 isolates (NZ007, NZ011, NZ014, NZ017, NZ052, NZ065, NZ096, NZ103, NZ104, NZ113) exhibited protease production, visible by halo formation (Table 4-2).

4.7.6 Extracellular lipase production

Extracellular lipase production was assayed qualitatively by growing BCOs on agar media (Appendix II) containing Tween™ 80 (Sierra Medium) and egg yolk (Egg yolk lecithin agar). Production of extracellular lipases results in an opaque precipitation emanating from around the colony. The basis for the enzymatic plate assays was the precipitation of calcium salts of free fatty acids produced from the substrates of Tween™ 80 by the action of lipases. As with protease production, lipase production is an easy phenotype to characterise. Furthermore, lipase production, and in particular phospholipase production has been associated as a contributing pathogenicity determinant in many bacteria including *Clostridium perfringens*, *Staphylococcus aureus* and *P. aeruginosa* (Titball, 1993, Songer, 1997). Furthermore, lipases have been identified in *P. tolaasii*, but their correlation with blotch disease is unsubstantiated (Baral and Fox, 1997). Of the 33 BCOs, nine (NZ011, NZI7, NZ039, NZ092, NZ096, NZ101, NZ102, NZ103, NZ104, NZ113) showed lipase visualised by a characteristic white precipitate whereas the remaining mutants were devoid of any lipase activity (Table 4-2).

4.7.7 Chitinase production

Chitinase production was assayed qualitatively by growing BCOs on media containing chitin extracted from crab shells (Chitinase plates, Appendix II). Chitinase production has been identified as a major pathogenicity determinant in pseudomonads involved with detrimental affect of fungi, including *P. gladioli* pv. *agaricicola* (Gill and Tsuneda, 1997) and contributes to the pathogenicity of

other characterised bacterial pathogens (Watanabe, *et al.*, 1997). However, of the 33 BCOs no isolates were observed to produce chitinase at detectable levels using this assay system.

4.7.8 Mushroom juice agar (MJA)

An important part of this section of work was to gain understanding into bacterial/fungal interactions and therefore, it was deemed important to develop a method for quick screening of the ability of an isolate to grow on only those nutrients provided by mushroom tissue. For this purpose, mushroom juice agar (MJA) was developed within this study. MJA was made by grinding 100 g of mushroom tissue (non-diseased, first flush) in 100 ml of ddH_2O with a mortar and pestle. The homogenate was then initially filtered through cheesecloth and then filter-sterilised using a vacuum bucheners flask containing a 0.2 μm membrane filter. A 500 ml solution of 2.2% agar was autoclaved separately, 200 ml of the MJ filtrate was combined, and agar plates were then poured.

Screening BCOs on MJA

All BCOs inoculated on MJA showed equivalent growth as on LBA, in respect to colony size and time to reach equivalent colony size. This observation is not all together surprising considering the nutrient analysis of mushroom tissues (Chapter 1.1.4) and the observation that all BCOs are prototrophic on M9 (Table 4-2).

4.8 Overview of BCOs pathogenicity determinants

The results of the phenotypic characterisation of BCOs are summarised in Table 4-2. Also provided in this table is a review of previously identified biochemical and phenotypic observations obtained in Chapter 2.

Table 4-2 Summary of BCO phenotypes. Isolates are categorised with respect to blotch formation, white-line reaction, API 20NE biochemical assimilation profile, biofilm, protease, lipase, auxotrophy, ability to grow on mushroom juice agar and siderophore production.

BCO	48hr	WL(R)	WL(T)	API NE 20	Biofilm	Protease	Lipase	M9	Mush J	PAF (fl)
NZ006	B6	-	-	0156555	-	-	-	+	+	+
NZ007	B4	-	+	0557555	++	+	-	+	+	+
NZ009	B3	-	+	1357555	+	-	-	+	+	+
NZ011	B6	-	-	0157555	++	+	+	+	+	+
NZ014	B3	-	-	1147555	++	+	-	+	+	+
NZ017	B3	-	-	4357555	++	+	+	+	+	+
NZ024	B3	-	-	1147455	++	-	-	+	+	+
NZ027	B9	+	-	0156555	++	+	-	+	+	+
NZ031	B4	-	-	0177575	++	-	-	+	+	+
NZ032	B9	+	-	0156555	++	+	-	+	+	+
NZ039	B3	-	-	0157555	++	-	++	+	+	+
NZ043	B5	-	-	0157555	++	-	-	+	+	+
NZ047	B5	-	-	0156565	++	-	-	+	+	+
NZ052	B9	-	++	0157575	++	++	-	+	+	+
NZ059	B6	-	-	0156555	++	-	-	+	+	+
NZ060	B4	-	++	0157575	+	-	-	+	+	+
NZ062	B9	-	-	0157575	++	-	-	+	+	+
NZ064	B1	-	-	0357555	-	-	-	+	+	+
NZ065	B3	-	+	0757555	-	+	-	+	+	+
NZ066	B1	-	-	0157555	+	-	-	+	+	+
NZ081	B1	-	-	0757555	-	-	-	+	+	+
NZ092	B5	-	-	0156555	++	-	++	+	+	+
NZ096	B2	-	+	0157555	+	+	+++	+	+	+
NZ097	B3	-	+	0356577	++	-	-	+	+	+
NZ099	B1	-	-	0357555	+	-	-	+	+	+
NZ101	B6	-	+	0166677	++	-	++	+	+	+
NZ102	B3	-	-	0357555	++	-	++	+	+	+
NZ103	B9	-	-	0157555	++	++	++	+	+	+
NZ104	B3	-	-	0157555	++	++	+++	+	+	+
NZ111	B9	-	-	0157577	+	-	-	+	+	+
NZ112	B3	-	-	4140457	++	-	-	+	+	+
NZ113	B3	-	-	0357555	+	++	+	+	+	+
NZ124	B8	-	-	0157555	++	-	-	+	+	+
PT 2192	B9	+	-	0156555	++	+	-	+	+	+
PT 1116	B9	+	-	0156555	++	+	-	+	+	+
PT 2325	B9	+	-	0156555	++	+	-	+	+	+
PG 3147	B5	-	-	0356555	+	-	-	+	+	+
PG 3146	B5	-	-	0356555	+/-	-	-	+	+	+
PR 1311	B2	-	+	0357555	+	-	-	+	+	+

BCO = isolate from Chapter 2
48hr = blotch discolouration observed after 48hr incubation on mushroom cube bioassay (Chapter 2.9)
WL(R) = white line observed when plated next to *P. reactans* NCPPB 1311
WL(T) = white line observed when plated next to *P. tolaasii* NCPPB 2192
API NE 20 = biochemical assimilation profile obtained using API NE 20 strip analysis (Chapter 2.12)
Biofilm = ability to form biofilm on PVC in L10 12 hr at 30°C (Chapter 4.7.1)
Protease = ability to cause proteolytic clearing of Milk Agar Plates (Chapter 4.7.5)
Lipase = ability to produce lipases and cause a precipitate on Sierra Medium (Chapter 4.7.6)
M9 = ability to grow on M9 medium (Chapter 4.7.3)
MJA = ability to grow on Mushroom Juice Agar (Chapter 4.7.8)
PAF = observation of siderophore production under UV (Chapter 4.7.4)

DISCUSSION

In any given research project, it is often impractical to explore all possibilities. Pathogenicity determinants are numerous amongst bacteria and it is acknowledged that many other pathogenicity determinants could have been investigated within this section of study. However, the pathogenicity determinants investigated were deemed representative of likely determinants of blotch based on previous studies of *P. tolaasii* and other pathogenic pseudomonads.

4.9 Analysis of BCO pathogenicity determinants

When reviewing the summary Table 4-2, it becomes apparent that all BCOs able to cause blotch, with the exception of biofilm formation[1], do not consistently produce any of the PPDs tested. As blotch discolouration remains the focal point to compare phenotypes with, even when trying to correlate a particular blotch discolouration (e.g. 'brown blotch', typically in the range of B6-B9) with an observed phenotype (e.g. lipase production) shows no consistency. An example of this would be protease production in which both NZ052 and NZ104 produce visibly equivalent amounts of protease, yet the isolates induce contrasting blotch discolourations of B9 and B3 respectively.

4.9.1 Biofilm formation by BCOs

Even though the 33 BCOs were pseudomonad species exhibiting evolutionary divergence (Chapter 2), all isolates were shown to be amenable to biofilm formation under at least one set of conditions used in this study. Previously established methodology in which biofilm formation was investigated using abiotic bioassays using microtitre dish plates, was easily transferred to this study. However, even given the standardised technique, parameters such as nutritional source, adhesion surface, incubation time and temperature were all varied to see their effect upon biofilm formation of respective BCOs.

Correlation of biofilm of BCOs on PVC with potential biofilms formed on the mushroom surface

As with any bioassay, caution must be placed in the assumptions made between observations *in vitro* and what is likely to occur *in situ*. It is well established that *in situ* environments are vastly dynamic and therefore replication in the laboratory can only expose organisms to limited conditions expressed in the environment. In this study, the assumption was made that phenotypes expressed within *in vitro* bioassays *may* contribute to the putative blotch development of mushrooms, but it is acknowledged that any laboratory phenotype is a result of the defined experimental parameters.

[1] Biofilm formation for certain BCOs required specific media and growth substrate (Table 4-1).

Therefore, biofilm on PVC surfaces may not necessarily indicate that biofilm formation occurs on the mushrooms *per se.* It does however, suggest that the BCOs have the ability to attach to a surface and grow as a community. Within this thesis (data not shown) attempt were made to show biofilm production on the mushroom surface by a) staining the mushroom cap with various bacterial stains and b) using GFP markers on stable plasmids and viewing by con-focal microscopy. The results of these assays showed that the mushroom tissue stains similar to bacterial cells, and also shows auto-fluorescence under con-focal microscopy. Preliminary investigation would suggest that red fluorescent protein (RFP) would be a worth while marker gene if further investigation of pseudomonads on *A. bisporus*.

Ability to grow in all differential types of media

Within biofilm assay results, it was shown that all 33 BCOs from Chapter 2 were able to produce biofilms, but only under specific media conditions (Table 4-1). That is, some isolates produced biofilms in: A) only L10 (a high nutrient media); B) only M9 (a minimal media); or C) both L10 and M9. For example, isolates NZ064 and NZ065 formed a biofilm in M9 but not in L10, whereas, isolates NZ007 and NZ096 formed a biofilm in L10 but not in M9. Interestingly, no BCO was unable to form a biofilm in both media[1]. However, the majority (29/33) produced biofilms in L10 media.

Similar to these BCO observations, assays for various other species of bacteria have shown preferences for types of media required for biofilm formation. *Vibrio cholerae* (Watnick and Kolter, 1999), *Staphylococcus epidermidis* (Heilmann, *et al.*, 1996a) and *E. coli* (Pratt and Kolter, 1998) utilised high nutrient media. In contrast, *Pseudomonas aeruginosa* PA14 (O'Toole and Kolter, 1998a), *Pseudomonas fluorescens* WCS365 (O'Toole and Kolter, 1998b), and *Streptococcus gordonii* (Loo, *et al.*, 2000) were primarily assessed for abiotic biofilm formation when grown in minimal media supplemented with glucose and casamino acids. Interestingly, some BCOs were not able to form biofilms in M9 minimal media whereas they could when grown in LB. Similar observations were made for *V. cholerae*, which also showed greatly decreased biofilm formation in minimal media (Watnick and Kolter, 1999). It is unclear whether the lack of biofilm formation of these BCOs in minimal media is a growth phase dependent phenomenon or due to the lack of a particular supplement required for activation of biofilm pathways. However, these observations support the notion that the nutrient requirements for biofilm formation may be quite specific and can vary between species.

Studies have shown that supplementation of media have rescued biofilm defective mutants of *P. fluorescens* (O'Toole and Kolter, 1998b), therefore, suggesting that different nutrients have the ability to activate different cellular mechanisms for biofilm formation. Examples such as these reinforce the substantial bias that can be placed upon experimental procedures by the simple choice of assay conditions influencing the type of mutations isolated. In the present study, it was decided that LB media be used in a 1:10 dilution with ddH_2O as LB greater mimics the highly nutritional environment

provided by the mushroom cap (Chapter 1.1.4) and furthermore, the greatest number of BCOs forming biofilms in this media was observed (Table 4-1).

Substrate specificity of BCOs

The biofilm substrate specificity of the BCOs provided another interesting point of comparison to other bacterial species. Some BCOs showed a preference of a particular surface for biofilm formation (Table 4-1). For example eleven BCOs were unable to grow on microtitre dishes made of polypropylene whereas they could on PVC[1]. All other BCOs were able to grow on at least one of the surfaces tested, however it was noted that certain surfaces facilitated formation of more heavily stained biofilms than others (Table 4-1). Polyvinyl chloride (PVC), polystyrene, polypropylene and borosilicate glass surfaces have been shown to support reproducible biofilm formation for *P. fluorescens* WCS365 (O'Toole and Kolter, 1998b) and *E. coli* 2K 1056 (Pratt and Kolter, 1998). The electrostatic forces associated with different surfaces are thought to be a significant barrier to initial colonisation (Razatos, *et al.*, 1998). PVC, polystyrene, and polypropylene are all relatively hydrophobic where as borosilicate glass is relatively hydrophilic. Adherence of BCOs to different surfaces may be facilitated by the ability to over come the repulsive forces associated with hydrophilic and hydrophobic surfaces. Research indicating that *V. cholerae* utilises different types of pili for attachment to an abiotic surface and chitin surfaces indicates the presence of distinct mechanisms for attachment to different substrates (Watnick, *et al.*, 1999). Furthermore, the authors proposed that *V. cholerae* may possess differential genetic pathways for attachment to different surfaces. It is most likely that different genetic regulatory processes and physiological states exist between the different BCOs which could account for the ability to attach to different surfaces. It was striking that all BCOs, even those that did not form blotch discolourations (NZ064, NZ066, NZ081, and NZ099) had the ability to form biofilms, albeit weak biofilms on selected surfaces. Although detailed exploration into this phenomenon was not carried out, it would prove a most interesting area for further investigation into the area of bacterial colonisation of diverse pseudomonads in the same environmental niche.

4.9.2 Extracellular compound production by BCOs

As well as biofilm formation, other extracellular compounds such as protease, lipase, and siderophore production were assessed. Such extracellular compounds have been shown to be contributing factors in the pathogenicity of other bacterial species and furthermore, these compounds have been identified in *P. tolaasii* and surmised to have involvement in brown blotch.

Lipodepsipeptide production

P. tolaasii tolaasin production is the primary cause of *P. tolaasii* brown blotch and although *P. reactans* has been described in a pathogenic form that causes small, dark purple spots on harvested

[1] Although specific surface substrates were required – discussed next section.

mushrooms (Wells, *et al.*, 1996), WLIPs are not considered as blotch causing agents and have even been used to prevent *P. tolaasii* brown blotch by sequestering tolaasin (Soler-Rivas, *et al.*, 1999b). All BCOs were tested in Chapter 2.11 for production of lipodepsipeptides that reacted with either *P. reactans* WLIP or *P. tolaasii* tolaasin as defined in the WLA assay (Wong and Preece, 1979). As discussed in Chapter 2.14.12, only eight isolates produced a WLA+ reaction to tolaasin, and two isolates produced a WLA+ to WLIP. Furthermore, this lipodepsipeptide production could not be correlated to a particular blotch discolouration phenotype (with exception of NZ027 and NZ032 which were considered homologous with *P. tolaasii*).

Protease and lipase production

Protease and lipase production has been identified in *P. tolaasii* (Baral, *et al.*, 1995, Baral and Fox, 1997) however, their involvement in brown blotch has been suggested but not substantiated. Extracellular production of proteases and lipases were analysed using previously described qualitative media containing respective substrates whose enzymatic breakdown can be visualised. Results indicated that both protease and lipase production was variably present amongst the 33 BCOs (Table 4-2) with some isolates producing neither (NZ006, NZ009, NZ024, NZ047, NZ059, NZ060, NZ062, NZ111, and NZ113), some produced both (NZ011, NZ017, NZ096, NZ104 and NZ113) and the remainder producing one of the two. Furthermore, some BCOs appeared to produce visibly more of a particular extracellular enzyme that was visualised by a strong agar plate observed phenotype (e.g. enhanced lipase production was visualised for NZ104 and NZ096).

Similar to lipodepsipeptide production, no correlation between one particular blotch phenotype with the presence/absence of extracellular proteases and lipases could be established. This disputed a hypothesis formed prior to undertaking this research that because membranes are primarily composed of proteins and phospholipids, BCOs may produce extracellular proteins that degrade these membrane constituents thus causing cellular disruption and ultimately blotch discolourations. Although the results in this study do not suggest the proteases and lipases produced by the respective BCOs are primarily responsible for causing blotch, it cannot be excluded that, similar to biofilm formation, different nutritional conditions play an important role in the regulation and expression of proteases and in the environment. This highlights a global problem of *in vitro* assay systems in that results may contain bias due to defined parameters selecting for a particular phenotype and overlooking possible *in situ* expression.

Siderophore production

Siderophore production was positively observed by all 33 BCOs investigated. This was not altogether surprising given that the BCOs were selectively isolated on Gould's media (Chapter 2.8) which contains selective agents for recovery of fluorescent pseudomonads (Gould, *et al.*, 1985).

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[1] Growth for NZ064 on PVC required M9 media.

Nevertheless, siderophore production was confirmed because iron acquisition is an example of a bacterium's ability to compete successfully with host tissue and normal flora for limited nutrients so it can survive and multiply. One of the most widely studied examples of this is the ability of bacteria to compete for iron acquisition via siderophore production (synthesised iron chelators capable of binding iron extracellularly). Often overlooked is the significant contribution that mechanisms that effect the rate of replication has on the ability of a particular bacterium to express pathogenicity and therefore, siderophore analysis was an acknowledgement of the involvement of such essential processes.

4.9.3 Growth on varying nutritional sources

Different media were used to determine fitness of the respective BCOs when grown under limiting nutritional availability. M9 minimal media was to assess the auxotrophic/prototrophic state of the BCOs and MJA was to determine growth on only those nutrients provided by *A. bisporus* mushroom tissue.

M9 minimal media

Further to the discussion within the above siderophore production, the ability of a bacterium to grow under adverse conditions furthers its ability to colonise and establish cell densities required to subsequently exhibit pathogenicity. Therefore, one could describe growth rates as a primary virulence factor. BCOs were screened on M9 minimal media (supplemented with glucose) to determine the prototrophic status. Given that mushroom tissue has been shown to contain many nutrients (Chapter 1.1.4) and in particular, provide a source of almost all essential amino acids (Beelman and Edwards, 1989, Foret and Arpin, 1991, Mattila, *et al.*, 2002) it was plausible that BCOs could have used mushrooms as an extracellular source of amino acids, and/or other nutrients if they were themselves deficient in their production (*i.e.* auxotrophic). However, all BCOs were found to be prototrophic and exhibited equal growth in comparison to each other. Having the ability to synthesise all essential amino acids and other cellular components in an environment is a physiological advantage. Although the selective composting procedures are designed for complex carbohydrate production provides a nutritionally rich environment for *A. bisporus* (Chapter 1.1.3), conditions for bacteria aren't so favourable. Simple carbohydrates are not as readily available, and furthermore after several flushes, any available nutrient levels will deplete among the exponentially multiplying bacteria. Therefore having the ability to grow in nutrient depleted environments is most likely an adaptive advantage required for bacterial population viability of any given BCO.

Mushroom juice agar (MJA)

Imperative to any study of a given environmental microorganism is the development of *in vitro* conditions that best replicate *in situ* conditions. Nutrient availability and culturing conditions have shown in many cases to effect phenotypic expression of different genes and therefore incorrect media

selection may bias investigations of particular bacterial traits. Mushroom juice agar (MJA)[1] was developed in this study to provide a medium that assessed the growth of BCOs with only those nutrients provided within the mushroom sporophore. Results showed that when BCOs were grown comparatively on MJA and LB, growth was equivalent. This was not an unexpected result as mushroom tissue contains most essential nutrients (Chapter 1.1.4) required for bacterial growth. Furthermore, as many BCOs were isolated from the surface of sporophores initially, it was likely that they would have had the adaptive cellular mechanisms to efficient replicate on solely on nutrients provided by the sporophore.

4.10 Selection of a single BCO (NZ103) for focused study

Because transposon mutagenesis was not feasible for all 33 BCOs, a single isolate was selected as a representative of the 33 BCOs for a focused transposon mutagenesis study described in the following chapter. As highlighted in Table 4-2, NZ103 was chosen because it produces: a) a strong mushroom tissue blotch discolouration (B9); b) a strong biofilm formation (Figure 4-3); c) produces an array of PPDs, including motility, protease production, lipase production, prototrophy, and siderophore production. Although PPDs (other than biofilm formation) were not consistently produced by all BCOs, it was considered beneficial to see their implication (if any) in the formation of blotch disease by NZ103 in the following chapter.

[1] Note that this was initially called 'mushroom extract agar', however, the acronym 'MEA' had already been assigned to 'malt extract agar' within our laboratory.

CONCLUSION

This chapter was a continuation from Chapter 2 in that it sought to elucidate what common pathogenicity determinants were present amongst BCOs. All 33 BCOs were tested for previously identified pathogenicity determinants, namely the production of: biofilms, motility, auxotrophy, siderophores, protease, lipase, chitinase, and the ability to grow on MJA. Observations were again similar to Chapter 2 in that no distinct phenotype revealed itself in this section of study and the 33 BCOs causing blotch discolourations of *A. bisporus* all appear to exhibit many variable phenotypes. The only truly consistent phenotypes observed were the abilities to grow on M9 minimal media and MJA agar and there were not unexpected phenotypes as prototrophy and the ability to utilise only those nutrients available in a given niche, are not only traits of pathogenicity, but also those required for evolutionary fitness and success of a species. Therefore, although these growth traits are an essential part of pathogenicity, they were not considered the sole reason why BCOs were causative of bacterial blotch. The only consistent phenotype other than growth on M9 and MJA was abiotic biofilm formation, however even this phenotype exhibited variability in parameters required for it development and furthermore, non-pathogenic BCOs were shown to form biofilms also. Therefore, it appears that results in this section have again shown the complexity of bacterial pathogenicity and suggest the ability to cause blotch is likely a result of a combination of many regulatory pathways, not a single pathogenicity determinant. A single pseudomonad, *P. putida NZ103*, was chosen for a detailed mutagenesis screen in the following chapter in which mutants would be tested for deficiencies in the PPDs assayed in this chapter and ultimately that of reduction of *A. bisporus* blotch discolouration. The aim of characterising such mutants was to give further insight into the mechanisms involved in why the presence of BCOs causes blotch discolouration of *A. bisporus* tissue.

Chapter 5

TRANSPOSON MUTAGENESIS OF NZ103 USING MINI-TN5KM*LACZ*2

As discussed in Chapter 4, isolate NZ103 was chosen in this section of study as a representative of the BCOs for mutational studies using the classic 'genes-to-phenotype' approach. The aim of this chapter was to identify genes involved in the expression of putative pathogenicity determinants (PPDs) in NZ103, such as biofilm formation; protease and lipase production; auxotrophy, and siderophore production. Presented is the selection of NZ103 mutants deficient in one or a number of these PPDs and the subsequent screening for reduction or loss in the ability to cause blotch discolouration in the mushroom tissue bioassay.

INTRODUCTION AND LITERATURE REVIEW

Many studies have used the classical 'genes-to-phenotype' approach to randomly 'knock-out' genes within bacterial chromosomes using transposons and then screen mutants for loss of a desired phenotype. Mutagenesis was chosen as a method of choice within this study to determine PPDs in BCOs as no prior information regarding PPDs was available. The major described pathogenicity factor of *P. tolaasii*, tolaasin, was shown to be absent amongst the BCOs (with exception of NZ027 and NZ032). Therefore, Chapter 4 set out to identify PPDs in the 33 BCOs identified in Chapter 2 and the only common PPD (with exception of prototrophy and siderophore production) amongst all 33 isolates was that of biofilm formation. Given the predicted genetic complexity of pathogenicity in pseudomonads, biofilm formation was likely to be only part of the cause of blotch disease by BCOs. Nonetheless, using the selected BCO, *P. putida* NZ103, Tn*5* mutagenesis was performed in order to generate mutants deficient in one or a number of the PPDs identified in Chapter 4, so as to ultimately assess their involvement in the blotch discolouration process.

5.1 Gene identification by random mutagenesis

In the following sections, an overview of previous research using mutagenesis approaches for the research of biofilms and other extracellularly produced compounds is presented.

5.1.1 Mutagenesis studies for identifying molecular mechanisms of biofilms

A common method for investigating the molecular processes involved in biofilms has been to generate mutants of the desired bacterium and assess their ability to form biofilms on abiotic surfaces. In this way, a correlation can be made between genes, their products, and the process of biofilm formation. Two different approaches can be used for mutational studies: 1) specific mutations are introduced into candidate genes and screened for their affect on biofilm formation; and/or 2) genes are randomly mutated and assessed for their involvement in biofilm formation, with no assumptions made regarding the nature of genes involved. Both approaches have provided useful information on the molecular mechanisms of biofilm formation in different organisms and are discussed within the following sections.

The studies carried out to date have encompassed a wide range of bacteria, some with closer taxonomic relationships than others. The natural niches that have undoubtedly shaped their evolution range from oral cavities for the Gram-positive bacterium *S. gordonii*, to the rhizosphere for the Gram-negative bacterium *P. fluorescens*. The emerging picture suggests that distinct mechanisms for regulating and mediating biofilm formation have evolved.

Some of the primary studies carried out under the directive to characterise genes involved in the initial stages of biofilm formation were those of O'Toole and Kolter. Mutants unable to form biofilms, referred to as surface adherent deficient (SAD) mutants, were isolated and partially characterised for both *P. fluorescens* (O'Toole and Kolter, 1998b) and *P. aeruginosa* (O'Toole and Kolter, 1998a).

Pseudomonas fluorescens

In the case of *P. fluorescens*, the screening of 14,000 transposon mutants led to the recovery of 37 SAD mutants (0.3%). Molecular characterisation of 24 of the 37 SAD mutants revealed that only three had insertions to genes of known function. This was suggested to indicate the ability of this approach to identify heretofore-unknown genes. One mutant had an insertion to the *clpP* gene, which is a subunit of the cytoplasmic Clp protease, however, its role in biofilm formation was not understood. The other two mutants had insertions in genes involved in flagella synthesis that agrees with their non-motile phenotype. Although largely uncharacterised, the SAD mutants served as tools to investigate the effect of the nutritional conditions of the media on biofilm formation. Interestingly, many of the SAD mutants were able to form biofilms when grown in minimal media with citrate or glutamate instead of glucose as the carbon source (O'Toole and Kolter, 1998b). The ability of these media to restore biofilm formation varied for the different SAD mutants. This finding was suggested as evidence for the existence of multiple convergent pathways for biofilm formation within *P. fluorescens*.

Pseudomonas aeruginosa

When the same screen was applied to *P. aeruginosa*, 15 SAD mutants were isolated from a pool of 2500 (0.6%) (O'Toole and Kolter, 1998a). Three of the SAD mutants were non-motile, one of which was shown to have an insertion in *flgK*, the flagella associated hook protein. The other major category of mutant was those with insertions in genes involved in type IV pili biosynthesis. Type IV pili have been associated with adherence and colonisation of eukaryotic surfaces as well as a surface associated movement known as twitching motility (Wall and Kaiser, 1999). This motility is distinct from that provided by flagella, such that type IV pili mutants show normal swarm patterns on motility agar. These two categories of mutants, Fla$^-$ and Pil$^-$, provided the tools to investigate the initial processes involved in biofilm formation. Specifically, the abiotic biofilm formation of *pilB*, *flgK* and wild type strains were followed over time using phase-contrast microscopy. This analysis showed that the wild type formed a monolayer of cells that over time actively associated to form microcolonies. In contrast, the *pilB* mutant could form a monolayer but was unable to form microcolonies, whereas the *flgK* mutant showed practically no adherence to the PVC over the course of the assay. From these results it was suggested that, for *P. aeruginosa*, flagella mediated motility was necessary for initial attachment and monolayer formation, whereas type IV pili were required for microcolony formation by enabling lateral movement across the surface. This study was one of the first to begin to correlate the molecular determinants of cellular structures with specific stages in biofilm formation and development.

Escherichia coli

Several studies have been undertaken to investigate the types of genes involved in the abiotic biofilm formation of *E. coli* (Genevaux, *et al.*, 1996, Pratt and Kolter, 1998, Danese, *et al.*, 2000). Using a similar system to that used by O'Toole and Kolter, Pratt and Kolter (1998) isolated 177 biofilm defective mutants, which accounted for 1.7% of the mutants screened. As with the previous pseudomonad studies, approximately half of these mutants had defects in flagella mediated motility. Previous research had already shown motility to be important in biofilm formation (de Wager, *et al.*, 1987, Fletcher, 1988, Korber, *et al.*, 1994), however these studies tended to utilise genotypically uncharacterised strains, and as such the specific functions of flagella mediated motility required for biofilm formation were ill-defined (O'Toole and Kolter, 1998a, Pratt and Kolter, 1998). Of the remaining mutants, most were localised to genes involved in the regulation or synthesis of type I pili. In a similar fashion to studies on *P. aeruginosa*, the biofilm formation of a type I pili mutant (*fim*H1) and a flagella mutant (Δ*mot*A) were compared to that of the wild type over time using phase contrast microscopy. In contrast to the results with *P. aeruginosa*, the pili mutant could not adhere at all, whereas the flagella mutant could form infrequent but distinct clusters of cells. These results suggest that although both flagella and pili are required for biofilm formation in *P. aeruginosa* and *E. coli*, their roles appear different. For *E. coli*, flagella mediated motility is thought to be required to overcome surface associated repulsion, and possibly mediate movement across the surface, whereas type I pili are required for attachment to the surface.

Further studies on the biofilm formation of *E. coli* have been carried out by Danese et al. (2000). This work reported the characterisation of a biofilm defective *E. coli* strain with an interruption to *wcaF*, a gene required for the production of the exopolysaccharide colanic acid. Unlike pili or flagella mutants, loss of colanic acid does not interfere with initial attachment, rather it appears to be involved in establishing the three dimensional structure of the biofilm. It is noted that the dense, tightly packed biofilm structure exhibited by the *wcaF* mutants is analogous to the biofilm phenotype of the *P. aeruginosa lasI* mutant. This correlation may be suggestive of a role for quorum-sensing in the regulation of colanic acid synthesis. Further links to colanic acid synthesis and biofilm formation were made in work by Prigent-Combaret et al. (1999) (Prigent-Combaret, *et al.*, 1999) demonstrating that the *wca* locus is upregulated upon attachment to abiotic surfaces.

Vibrio cholerae

The study of *Vibrio cholerae* biofilm formation resulted in the isolation of randomly generated biofilm defective mutants (Watnick and Kolter, 1999) and as previous Gram-negative bacterial studies, mutations were isolated in genes required for motility, type IV pili (MSHA), and exopolysaccharide production.

Staphylococcus epidermidis

Biofilm formation is thought to contribute to the virulence of *Staphylococcus epidermidis* which is a common cause of implant associated infections (Heilmann, *et al.*, 1996a). The current model for *S. epidermidis* biofilm formation involves two major stages; rapid primary attachment to the polymer surface, followed by a slower accumulation of cells into multi-layered clusters. The molecular mechanisms that mediate these processes have been the subject of significant investigation. A mutagenesis screen reported the recovery of four mutants out of 5000 that were unable to form biofilms on polystyrene (Heilmann, *et al.*, 1996a). This equates to a 0.08% recovery, a proportion that is markedly lower than in the studies on Gram-negative bacteria referred to above. In line with the two step process suggested for biofilm formation, two classes of mutant were defined; those unable to attach, and those that could attach but were unable to form cell aggregates. Noticeably absent from this model of biofilm formation is the involvement of motility to either overcome surface associated forces or for movement across the surface. It would seem that cell clusters derive from cell division and intercellular adherence rather than active movement of individual cells. One of the mutants demonstrating defects in primary attachment was shown to have a disruption to *altE*, an autolysin with cell wall hydrolytic activities (Heilmann, *et al.*, 1997). This is the first report suggesting a role for an autolysin in mediating attachment with inert surfaces. Subsequent reports have shown that one of the mutants unable to form cellular clusters has an insertion to a locus responsible for the production of a polysaccharide intercellular adhesin (PIA) (Heilmann, *et al.*, 1996b). Purification of this PIA has shown it to be a novel glucosaminoglycan and work is now in progress to elucidate its biosynthetic pathways (Mack, *et al.*, 1996).

Streptococcus gordonii

Another Gram-positive bacterium investigated with respect to characterising the genes involved in biofilm formation was *Streptococcus gordonii* (Loo, *et al.*, 2000). Out of 25,000 transposon mutants 18 (0.07%) were designated as defective for biofilm formation. The authors report that biofilms associated with these mutants ranged from 39% to 91% reduced relative to wild type biofilm formation. One mutation of interest mapped to *comD* (part of the competence regulon), the sensing component of a two-component signal transduction system involved in density dependent regulation. These findings agree with those of Davies et al. (1998) and suggest that the importance of cell to cell signaling in biofilm formation is not restricted to Gram-negative bacteria.

Summary of biofilm research

Focused research into bacterial biofilms has shown they are highly organised structures comprised of complex metabolic and physical interactions of bacteria within an intricate three-dimensional structure; thus biofilms have been described as a 'city of microbes' (Watnick and Kolter, 2000). In recent years the advent of molecular developments have provided insight into biofilm formation of different bacterial species. From these studies it is clear that biofilm formation is a highly regulated

and complex process at the molecular level. Not only does biofilm formation require the expression of multiple genes, such expression is highly coordinated in response to specific environmental signals (O'Toole, *et al.*, 2000a, Monds, *et al.*, 2001). Furthermore, it is becoming increasingly apparent that different sets of genes are required for distinct phases of biofilm formation, such as attachment, microcolony formation, biofilm differentiation and biofilm degradation. In this way, biofilm formation is now being viewed as a multicellular developmental process (O'Toole, *et al.*, 2000a).

Molecular investigations of biofilm formation are continually identifying novel roles for previously characterised genes, as well as previously undescribed genes. This is certainly one of the main utilities of a random mutagenesis approach. In many cases, the role that such genes play in biofilm formation is not obvious. In this respect, it is becoming apparent that the molecular processes involved in regulating and effecting biofilm formation are more integrated, complex and diverse than was first conceived. The current research has been carried out with an aim to contributing to the growing body of knowledge on the molecular mechanisms of biofilm formation.

5.1.2 Mutagenesis studies of extracellularly exported compounds

As well as being pivotal for the understanding of the molecular basis of biofilm formation discussed above, mutagenesis studies have been invaluable in the elucidation of the genetic basis of other bacterial pathogenic determinants.

Protease and lipase production

Characterising extracellular heat-stable proteases and lipases produced by psychrotrophic pseudomonads have been studied extensively (Fairbairn and Law, 1986, Stead, 1986), however, most of these studies have focused on *P. fluorescens* strains associated with spoilage of milk and diary products (Stepaniak, *et al.*, 1982, Fairbairn and Law, 1986, Mitchell and Marshall, 1989). Studies using Tn*5* transposon mutagenesis have been carried out to examine the molecular regulation of extracellular enzyme production in which mutants deficient in protease production where identified (Liao, *et al.*, 1997) and then further investigated (Liao and McCallus, 1998). Lipases and proteases are often observed to be jointly regulated. An example of this is in *P. brassicacearum* in which three enzymes, lipase, a serine protease homolog and an extracellular alkaline protease are encoded in an operon and are all under the same regulation during phase variation (Chabeaud, *et al.*, 2001).

5.2 Objectives for Chapter 5

Using the selected BCO, *P. putida* NZ103, this section of study was undertaken to randomly mutate NZ103 in order to obtain mutants deficient in one or many of the PPDs determined in Chapter 4 and ultimately determine whether mutants in these PPDs had an effect on blotch discolouration.

Although the only common determinant amongst the BCO isolates was that of biofilm formation, interest was still placed in the other PPDs such as prototrophy, siderophore production, protease and lipase production. Therefore, the objectives for this section of work were:

1. To randomly insert the transposon, mini-Tn*5*km*lac*Z2, into the chromosome of the selected BCO isolate, *P. putida* NZ103.
2. To identify NZ103 Tn*5* mutants that are deficient in one or a combination of the pathogenicity determinants described in Chapter 4:
3. To assess the involvement of the selected pathogenicity determinants in blotch disease using the *in vitro A. bisporus* mushroom cube bioassay.

METHODS AND RESULTS

5.3 Transposon mutagenesis of NZ103 using mini-Tn*5*km*lacZ2*

The transposon mini-Tn*5*km*lacZ2* was used to generate a collection of random insertional mutations in NZ103. Mini-Tn*5*km*lacZ2* is a derivative of *Tn5* (Simon, 1989) modified specifically for use as a mutagenic agent. The transposase is supplied in trans on a pUT suicide delivery vector that requires the p protein for replication (de Lorenzo, *et al.*, 1990). The effect of this modification is to reduce the probability of a second transposition event and thus increase the stability of the mutation. The mini-Tn*5*km*lacZ2* also contains a *lacZ* gene that lacks transcriptional or translational signals, and can therefore be used to create protein fusions. A kanamycin (Km) cassette provides antibiotic selection for the transposon (Figure 5-1).

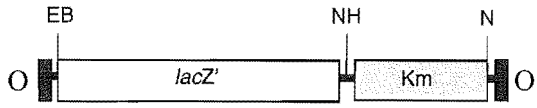


Figure 5-1 Schematic depiction of mini-Tn*5*km*lacZ2* (de Lorenzo *et al.*, 1990). Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III, O, IS50.

5.3.1 Optimisation of conjugation of NZ103 with mini-Tn*5*km*lacZ2*

Two parameters were optimised in an effort to maximise the efficiency of a large scale mutagenesis screen of NZ103: (i) optimisation of the donor to recipient ratio to obtain the highest number of transformants for a given incubation time; and (ii) the shortest length of incubation required for conjugation that still gave rise to around 100-150 transformants. Determination of these parameters increased efficiency by reducing the number of clonal transformants that were screened. A donor to recipient ratio of 1:4 was found to provide the highest frequency of transformation and by utilising this ratio, incubation times for conjugation were reduced to one hour while still obtaining the desired numbers of transformants.

5.3.2 Mini-Tn*5* mutagenesis and screening for phenotype deficient strains

Mini-Tn*5*km*lacZ2* was used to generate approximately 5000 random NZ103 insertional mutants (as described in Appendix 8.7I(xii)) originating from ca. 66 separate conjugations.

Replica plating

Kanamycin resistant NZ103 colonies arising as a result integration of mini-Tn*5*km*lac*Z2 were toothpick transferred onto LBA supplemented with Km (to select for Tn*5*). Colonies were spatially arranged on the agar plate according to a 48 grid pattern (Figure 5-2 A) This grid was designed to replicate half of a 96 well microtitre plate. A sterilisable aluminum replica-plater was designed (Figure 5-2 B) to enable multiple replication of numerous colonies grown in this pattern.

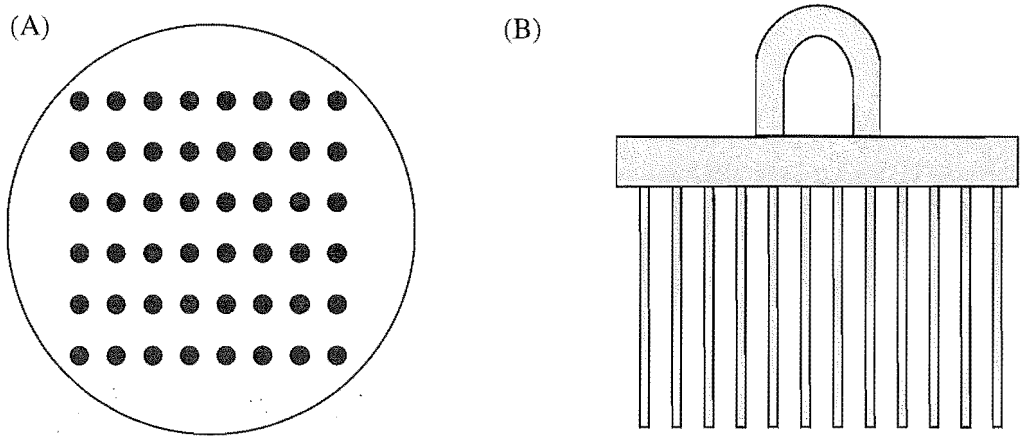


Figure 5-2 (A) The 48 grid pattern in which colonies were toothpick transferred on respective 75mm Petri Dish agar plates. (B) the replica-plater designed in this study for multiple transfers of colonies arranged as in A).

Following incubation and assignment of a colony code reference, colonies containing mini-*Tn5*kmlacZ2 (Km resistant) were replica plated onto agar plates supplemented with various indicator substances to examine the range of phenotypes described in the following sections. In these phenotypic analyses, NZ103 mutants were assessed for any deviation in phenotypic expression relative to the wild type NZ103. These assays are outlined below.

5.4 Phenotypic characterisation of NZ103 Tn5 mutants

The genetic basis for pathogenicity determinants is a complex phenomenon. It is likely that blotch requires the coordination of multiple cellular responses by global regulatory mechanisms. Because of this, transposon insertional mutations of NZ103 may affect multiple genetic pathways, and as such, a number of different PPDs were tested as outlined in Chapter 4. Please note that only selected individual mutants are described (if appropriate) in the following sections addressing individual phenotypic analyses. However, a tabulated summarization of all relevant mutants is presented following all analyses to allow comparison of phenotypic deficiencies (Table 5-1).

5.4.1 Abiotic biofilm formation

Optimal conditions for the *in vitro* biofilm assay were determined for NZ103 (based on preliminary NZ103 assays, Chapter 4.7.1) and defined as PVC surface, 12 hours growth at 30°C in L10 inoculated with a 1:50 dilution from a stationary phase culture. Out of the ca. 5000 NZ103 mutants screened, 37 were recovered that had a reproducible biofilm-deficient phenotype. This equates to 0.47% of the total number of mutants generated. All 37 mutants had a very stringent minus phenotype, in that wells were completely devoid of ring formation. In addition to the biofilm defective mutants (Bfm-), six Bfm- reduced and one biofilm enhanced mutant (NZ103 B5++) were recovered (Figure 5-3). All mutants identified in the first round of screening were re-analysed using the abiotic *in vitro* assay a further three times to ensure reproducibility of biofilm altered phenotypes.

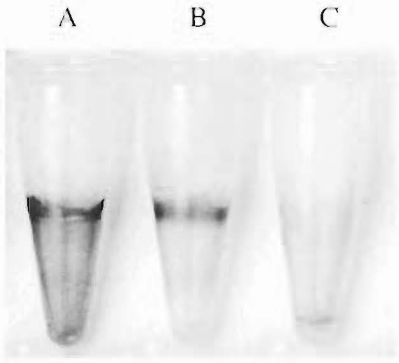


Figure 5-3 Biofilm phenotypes of biofilm mutants. Representative biofilm phenotypes after 16 hours growth of (A) a Biofilm enhanced mutant (NZ103-B5++); (B) NZ103 wildtype; and (C) a biofilm defective mutant (103G3).

5.4.2 Flagella mediated motility assays

The motility of NZ103 biofilm mutants were initially characterised by using motility agar and those mutants deficient were subsequently analysed by transmission electron microscope (TEM).

Motility agar analysis

Motility agar analysis was performed and scored as previously discussed (Chapter 4.7.2). All 14 biofilm-altered NZ103 mutants selected within Table 5-1 were analysed and all 14 displayed a motile phenotype comparable to NZ103 wildtype. Also presented in Table 5-1 are biofilm positive mutants that did exhibit altered motility phenotypes: these five mutants (103 G29, D25, B26, D24 and G21) were found to be completely non-motile based on the definitions given in Chapter 4.7.2 and a further five mutants (103 E23, C27, F26, G13 and E10) displayed an altered pattern of motility compared to that of the wild type (Figure 5-4). These mutants are interesting in that they have obvious motility deficiencies, but the cells are still able to form efficient abiotic biofilms in the conditions defined in this study. The biofilm-enhanced candidate, NZ103 B5++, had a motility pattern indistinguishable from that of NZ103 wild type.

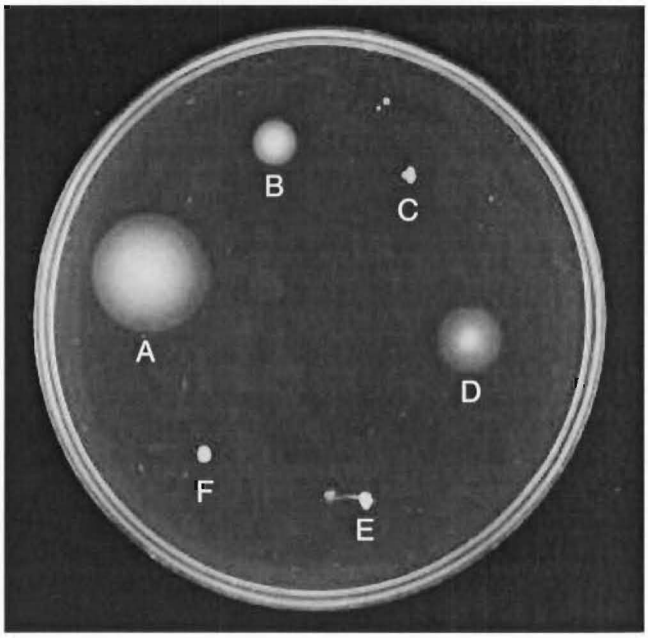


Figure 5-4 Representative flagella motility phenotypes of biofilm mutants. All strains are shown after 24 hours incubation on 0.3% motility agar plates: (A) wildtype swarm pattern NZ103; reduced motility patterns of: (B) 103F26; (D) 103C27; and non-motile phenotypes of (C) 103C29, (E) 103E23, and (F) 103 D24.

Transmission electron microscope analysis (TEM) of motility impaired mutants

A multitude of different mutations can give rise to a non-motile phenotype (Macnab, 1992). The different types of mutations do however fit into broad categories in terms of their effect on flagella biogenesis and function. Mutations affecting flagella biogenesis prevent complete flagella formation whereas mutations affecting torque generation lead to flagella paralysis but do not interfere with flagella biosynthesis.

In order to discern between these two possibilities, the flagellation phenotype of mutants that displayed abnormal motility patterns were examined using TEM. Firstly, NZ103 (wildtype) was examined and found to be a rod shaped bacterium with multiple[1] polar flagella originating from a single end of the cell along with other 'pili-like' structures[1] (Figure 5-5A). Also apparent was the consistent 'clumping' of cells with one another and a darkly stained extracellular matrix between these 'clumped' cells (Figure 5-5B). This outer staining was considered consistent with extracellular polysaccharides (EPS) often associated with fluorescent pseudomonads (Fett, *et al.*, 1995).

[1] Number of flagella were difficult to count because numbers varied amongst bacteria under the same TEM preparation. However, typically there were from between 4-8.

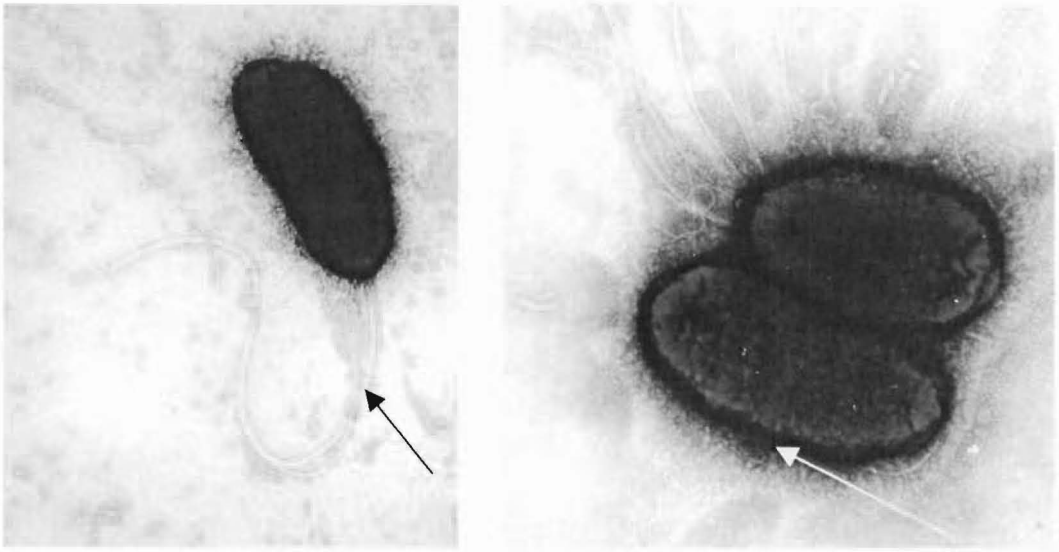


Figure 5-5 TEM analysis of NZ103 wildtype cell physiology. (A) Multiple polar flagella are visible from the rod shaped bacterial cell with pili like structures were also visible from the polar region of the cell. (B) The dark staining surrounding cells is considered to represent extracellular polysaccharides.

TEM was further used to examine the nine NZ103 mutants with motility altered phenotypes (NZG13, G21, F26, D24, B26, C27, D25, C29, E23) (Table 5-1). Observation of mutants deficient in motility (NZG21, D24, B26, D25, and C29) revealed that they had an absence of the multiple polar flagella seen in the NZ103 WT, however they did retain the 'pili-like' structures from the polar end of the cell. Mutants showing a reduced motility phenotype with altered growth pattern (103G13, F26, C27 and E23) were shown to still have polar multiple flagella visible comparable to NZ103 wildtype.

5.4.3 TEM analysis of selected biofilm deficient mutants

TEM was used to determine whether any differences between NZ103 wildtype and the biofilm altered mutants existed, as many previous studies have correlated biofilm loss with flagella loss. Although motility assays above suggest that motility has not been affected by the mini-Tn*5*km*lac*Z2 insertion, TEM was still used on selected biofilm negative mutants to ascertain whether any other gross-cellular physiology had changed.

Mutants 103A5, 103G3, 103F2, 103E2 and 103D2 were arbitrarily chosen for TEM investigation. The first interesting observation is that these mutants suspended into ddH_2O very easily compared to NZ103 wildtype and other biofilm positive mutants (such as motility negative, biofilm positive mutants described above) that formed clumps of cells. TEM observation of 103A5, 103G3, 103F2, 103E2 and 103D2 showed that these cells were different to NZ103 wildtype cells in that the cell shape

[1] These structures warrant follow up as they sound similar to the 'filament-like appendages' identified as involved in attachment to *A. bisporus* hyphae observed previously in SEM and TEM (Masaphy *et al.* (1987)).

was more elongated and the degree of staining of the cell wall was noticeably less than NZ103 wildtype (figure 5-7).

5.4.4 TEM analysis of biofilm enhanced mutant 103B5++

103B5++ was of particular interest because it produced an enhanced biofilm phenotype. When 103B5++ cells were observed by TEM, individual cell-morphology was not strikingly different to NZ103 wildtype, however, observation of the juxtaposition of cells revealed that more of the darkly stained EPS was visible between cells, which was suggestive of more EPS being produced by 103B5++ than NZ103 wildtype cells (figure 5-7).

5.4.5 Auxotrophy

All NZ103 mutants were screened for auxotrophy by assessing their ability to grow on M9 minimal media (M9, Appendix II). Of the 5000 mutants, 34 (0.7%) were found to be auxotrophic, while a further 71 (1.4%) exhibited very poor growth on M9 (Table 5-1). When combined, a total of 105 (2.1%) exhibiting deficiencies in prototrophic status agrees roughly with what has been reported for previous mutagenesis screens of other pseudomonads (Carruthers *et al.*, 1994) and suggests that mini-Tn*5*km*lacZ2* has inserted into target DNA randomly. Furthermore, mini-Tn*5*km*lacZ2* is a second generation Tn*5* derivative that has been optimized to provide random insertion and has been used in numerous studies with great success (de Lorenzo *et al.* 1990).

5.4.6 Siderophore production

Fluorescent siderophore production was assayed qualitatively by growing mutants on the iron limiting media, Kings media 'B' and *Pseudomonas* minimal media (PMM) (Appendix II). All 45 mutants in Table 5-1 exhibited siderophore production comparable to the wild type.

5.4.7 Extracellular protease production

Extracellular protease production was assayed (as previously described in Chapter 4.7.5) qualitatively on Milk Agar (Appendix II) and of the 5000 NZ103 mutants, 12 (0.2%) isolates (Table 5-1) exhibited a protease deficient phenotype (with no halo formation) whereas 3 exhibited a reduction in protease production comparable to the wild type (measured by millimeters of zone clearing) (Table 5-1).

5.4.8 Extracellular lipase production

Extracellular lipase production of NZ103 mutants was assayed qualitatively (as previously described in Chapter 4.7.6) by growing mutants on agar media containing Tween™ 80 (Sierra Medium, Appendix II). Of the 5000 mutants, 11 (0.2%) NZ103 mutants showed reduced extracellular

lipase activity (Table 5-1) that was visible by a reduction of the amount of precipitate formation comparable to NZ103 wild type (measured by millimeters of precipitation) (Table 5-1).

5.4.9 Growth on MJA

All mutants were shown to grow on MJA equally well as NZ103 wildtype (determined by colony diameter and rate of growth) with exception of 11 isolates that showed reduced growth rates (Table 5-1), and one isolate, 103F5, showed no visible growth on MJA.

5.4.10 Determination of the ability of NZ103 mutants to induce blotch

Ultimately, determining the ability or otherwise of NZ103 mutants to cause blotch was the most important phenotype within this chapter. Because the *in vitro* mushroom-cube assay is labour intensive and time consuming, it was unrealistic to screen all 5000 mini-Tn*5*km*lac*Z2 mutants for their blotch causing phenotype. Therefore, results of the putative phenotypic analysis presented above were analysed and 45 mutants deficient in one or a combination of the candidate pathogenicity determinants were selected (Table 5-1) and subjected to *in vitro* mushroom cube tissue bioassays (as previously described in Chapter 2.9). Results of the degree of blotch discolouration caused by each of these mutants is presented in Table 5-1. Blotch scale was determined as described in Chapter 2.9 and the assigned scale in Table 5-1 is the result of four independent bioassays to ensure reproducibility. Similar to observations in Chapter 2, blotch discolorations were invariably within one blotch scale in difference either side of ultimately determined blotch averages. Results of the mushroom cube bioassay showed that 12 (27%) of the 45 selected mutants exhibited wildtype blotch (B7-B9 phenotype), 20 (44%) showed a reduction in blotch (B4-B6 phenotype) and the remaining 13 (29%) had substantial reduction in blotch formation (B1-B3 phenotype).

5.4.11 Growth analysis of pathogenicity determinant deficient strains

The possibility existed that the inability of a NZ103 mutant to produce a given phenotype was due to severe growth defects associated with the mutation rather than interruptions in the genetic loci involved in the expression of the desired pathogenicity determinant *per se*. For this reason, the growth of selected NZ103 mutants was sought. Due to the large number of mutants, only low-resolution growth data was obtained for selected mutants (103G3, 103F3, 103A5, 103F5, 103A14, 103D13, 103A3, 103D2, 103F2, 103B5, 103C27) and was compared to that of NZ103 wild type. Inoculated LB broth cultures were grown and OD_{600} readings were determined at 0, 2, 4, 6, 12, 24, 36 and 48 hrs (as described in Appendix I(i)c).

Table 5-1 The 45 NZ103 mutant phenotypes selected to undergo mushroom-cube bioassays. Mutants are categorised with respect to their phenotypes.

Strain	Biofilm	Lipase	Protease	M9	Mush J	Motility	Blotch Scale*
103 WT	+	+	+	+	+	+	B9
103 A1	-	-	-	+	**R**	+	B5
103 B1	**R**	-	-	+	**R**	+	B4
103 D1	-	+	+	+	**R**	+	B2
103 E1	-	+	+	+	**R**	+	B5
103 G1	**R**	+	+	+	+	+	B5
103 A2	+	+	+	**R**	+	+	B6
103 C2	-	+	+	+	+	+	B5
103 D2/A10*	-	-	-	+	**R**	+	B3
103 E2	-	-	-	+	**R**	+	B1
103 F2	-	-	-	+	**R**	+	B2
103 G2	-	-	-	**R**	**R**	+	B1/B2
103 A3/A15*	-	-	-	+	+	+	B2
103 E3/B10*	+	+	-	+	+	+	B6
103 F3	+	+	+	-	+	+	B3
103 G3/B15*	-	-	-	**R**	**R**	+	B1
103 D4	+	+	+	+	**R**	+	B4
103 G4/D12*	-	+	**R**	**R**	+	+	B9
103 A5/D10*	-	+	-	+	+	+	B2
103 B5	+++	+	+	+	+	+	B7
103 C5	+	+	+	+	+	+	B7
103 D5	+	+	+	+	+	+	B9
103 F5/F12/F15*	+	+	+	+	-	+	B3
103 33*	+	+	+	+	+	+	B8
103 A13	+	-	+	+	+	+	B7
103 A14	+	-	-	+	+	+	B3
103 C13	+	+	-	+	+	+	B6
103 D11	+	+	**R**	+	+	+	B5
103 D13	+	-	+	+	+	+	B4
103 D14	+	+	+	-	+	+	B5/B6
103 E10	+	+	+	-	+	**-R**	B3
103 E11	+	+	+	-	+	+	B4
103 E15	+	+	+	-	+	+	B7
103 F14	+	+	**R**	-	+	+	B8
103 G10	+	+	+	-	+	+	B8
103 G11	+	+	+	-	+	+	B4
103 G12	+	+	+	-	+	+	B6
103 G13	+	+	+	++	+	**-R**	B7
103 G21	+	+	+	+	+	-	B6
103 F26	+	+	+	+	+	**R**	B3
103 D24	+	+	+	+	**R**	-	B4
103 B26	+	+	+	+	+	-	B4
103 C27	+	+	+	+	+	**R**	B4
103 D25	+	+	+	+	+	-	B7
103 C29	+	+	+	+	+	-	B5
103 E23	+	+	+	+	+	-	B9

- Does not exhibit the selected phenotype
+ exhibits the phenotype comparable to wild type NZ103
+++ exhibits the selected phenotype more than wildtype NZ103
R growth is reduced compared to wildtype NZ103
* the denotes suspected clonal strains as phenotype and selection from the same library pool

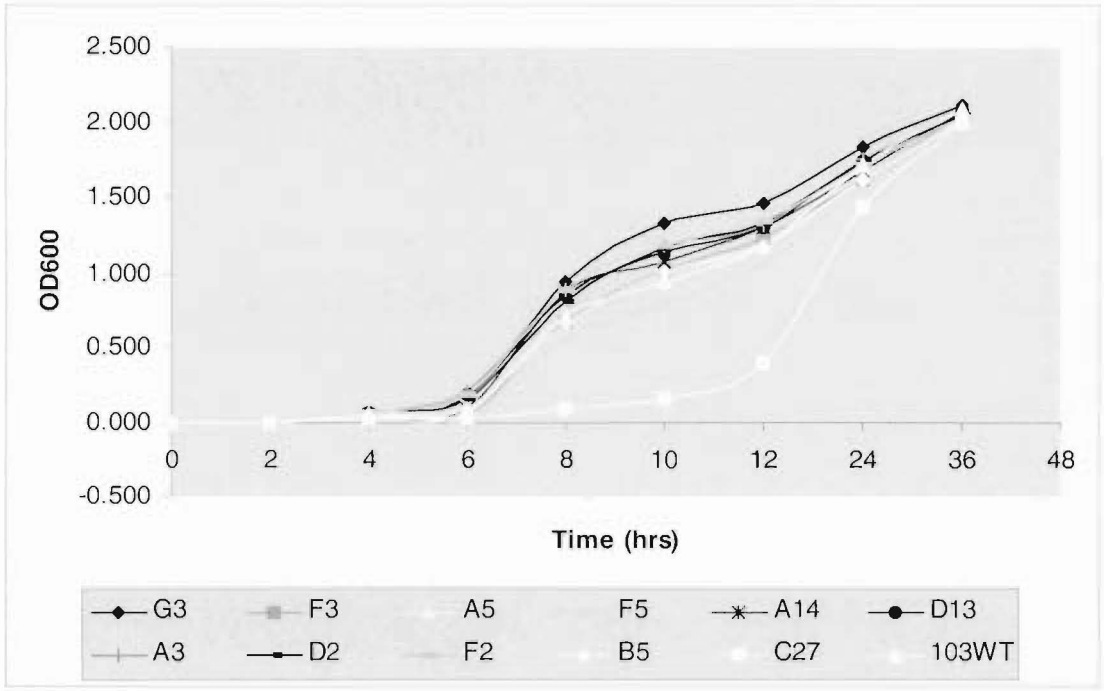


Figure 5-6 Low-resolution growth analysis of mini-Tn5km*lacZ*2 NZ103 mutants in which spectrophometer OD_{600} readings were taken at 0, 2, 4, 6, 8, 10, 12, 24, 36, and 48hrs for the respective cultures. Standard error was calculated from three independent replicates.

Of the eleven mutant strains evaluated for growth defects in LB, only two strains (103F5 and 103C27) exhibited reduced growth rates compared to NZ103 during the exponential phase (Figure 5-6), but it was noted that after 24 hrs cell density was comparable. The remainder of the NZ103 mutants grew to levels similar to NZ103 wildtype. It is worth noting that some mutants actually appeared to grow slightly better than NZ103WT, in particular 103G3. These results suggest that blotch reductions caused by these mutants tested was not a result of a defective growth rate[1] and that the mutation may have directly affected the ability to grow on mushroom cube nutrients and/or cause blotch discolourations.

[1] It is recognised that media choice may mask the deficiency of growth-rate.

DISCUSSION

NZ103 is a blotch causing pseudomonad that was identified because it had colonised a mushroom farm environment and was subsequently shown to cause blotch discolourations of *A. bisporus*. The blotch inducing properties of NZ103 have not yet been established, and therefore as a means of identifying and characterising genes that were involved in blotch formation, a random mutagenesis screen of *P. putida NZ103* was performed and mutants that were defective for selected PPDs were recovered (Table 5-1). It was shown that from a screen of approximately 5000 mini-Tn*5*km*lacZ*2 generated NZ103 mutants; several had phenotypes that were deficient in one or a combination of PPDs. Forty-five of these mutants were selected and screened using *in vitro* mushroom cube bioassays and of these, 20 (44%) showed a reduction in blotch (B4-B6 phenotype) and 13 (29%) had substantial reduction in blotch formation (B1-B3 phenotype) (Table 5-1). Such mutants were termed BCO^R mutants for discussion purposes.

The structure of this discussion will initially focus on independent phenotypic results observed within this section of study as a result of insertional mini-Tn*5*km*lacZ*2 mutagenesis. Furthermore, a correlation of these phenotypic deficiencies, with each other, and with the degree of blotch discolouration will be analysed.

The use of mini-Tn*5*km*lacZ*2 to generate NZ103 mutants

Using the mini-Tn*5*km*lacZ*2 as a mutagenic agent was, on the whole, an efficient method of generating NZ103 mutants. The only limitation foreseen was establishing rigorous screening methods for the desired phenotype(s), that is, the PPDs selected within Chapter 4. As Tn*5* mutagenesis generates thousands of mutant isolates, tests that enable high throughput with minimal effort are preferentially chosen. Therefore, PPDs determined in Chapter 4 were chosen for two reasons: (i) these determinants were considered to be highly likely to be involved in blotch formation; and (ii) these phenotypes were amenable for development of high throughput systems. With the construction of the replica-plater used in this study (Figure 5-2B), high throughput of microtitre dish and agar plate inoculation was possible with minimal effort. The only phenotype that could not have high throughput analysis was that of blotch determination using the *in vitro* mushroom cube bioassay. As such blotch determination was only performed on the 45 isolates exhibiting deficiencies in one or a combination of other phenotype identified (Table 5-1).

5.5 Mutants exhibiting biofilm altered phenotypes

As was established in Chapter 4, biofilm formation was the one consistent phenotype amongst the 33 BCOs isolated within Chapter 2 and therefore, interest was placed on mutants displaying altered biofilm phenotypes as a result of this mini-Tn*5*km*lacZ*2 screen.

Biofilm formation by NZ103

When reviewing literature on biofilms[1], it is possible to identify three areas that are important components of biofilm formation: (i) extracellular appendages such as flagella and pili play an important role in initial attachment and microcolony formation in a number of bacterial species, however, the specific role they play varies between organisms and in different environmental conditions; (ii) the production of specific exopolysaccharides is implicated as the scaffolding of biofilm architecture; and (iii) cell to cell signaling between bacteria within biofilms is required to mediate coordinated responses. When comparing these areas of importance to investigations of NZ103 mutants presented within this chapter, certain comparisons emerge: (i) NZ103 flagella are not a necessary component of biofilm formation within the context of the *in vitro* assay; (ii) exopolysaccharides in NZ103 appear important to biofilm formation; and (iii) the identification of 103B5++ suggests that cell-to-cell signaling may be involved in biofilm formation. These observations are discussed within the following sections.

Total number of biofilm mutants recovered from this study

As a result of the 5000 NZ103 mutants generated in this study, 37 mutants demonstrated a biofilm deficient phenotype and six demonstrated a biofilm-reduced phenotype. The complete loss of ring formation on PVC wells at the oxygen/media interface was used as the criteria for selection of biofilm minus mutants, and a reproducible reduction in ring formation (comparable to NZ103 wildtype) defined a biofilm-reduced phenotype. Putative biofilm mutants with severe growth defects described in other studies have tended to be discarded as genuine candidates at an early stage of investigation (O'Toole and Kolter, 1998b, O'Toole and Kolter, 1998a, Pratt and Kolter, 1998, Watnick and Kolter, 1999, Loo, *et al.*, 2000). It is important to note that a mutation affecting growth is not excluded from also affecting a specific pathway for biofilm formation distinct from its effect on the bacterium's growth rate. However, discerning between these two effects would be experimentally difficult.

5.5.2 Involvement of EPS in biofilm formation of 103B5++

One mutant recovered from the biofilm assay exhibited a biofilm-enhanced phenotype[2] and was of special interest as it suggested that biofilm formation is under regulatory control and the mini-Tn*5*km*lacZ*2 had inserted into a region that had enabled enhanced biofilm formation. One noticeable

[1] Presented in the introduction of this chapter, section 5.1.1.
[2] That is the biofilm ring stained more intensively than that of NZ103 wildtype biofilm (Figure 5-3)

phenotypic difference of 103B5++ was its mucoidy colony, and was consistent with the isolation of *P. fluorescens* mutants that have enhanced attachment properties and were also shown to either inhibit biosynthesis of lipopolysaccharide (LPS) or lead to over expression of an exopolysaccharide (EPS) has been described (O'Toole, *et al.*, 2000a). TEM observation of an enhanced staining between 103B5++ 'clumped' cells is highly suggestive of an overproduction of EPS in comparison to NZ103 wildtype. When considering the role of EPS production by BCOs, it may play an important role in both the stability and vertical development of biofilm formation (Costerton, *et al.*, 1987) as well as the survival in the mushroom cultivation environment by protecting the bacteria from adverse environmental conditions such as desiccation (Singh, *et al.*, 1992, Osphir and Gutnick, 1994). Furthermore, it has been established that EPSs are synthesised by fluorescent pseudomonads in the mushroom environment (Fett, *et al.*, 1995) and that these polymers mediate bacterial attachment to fungal mycelia (Rainey, 1991).

It is possible that the mini-Tn*5*km*lac*Z2 in 103B5++ has inserted into a gene that is responsible for the regulation of EPS production, however, because no other obvious phenotypes were eliminated, the gene interrupted may encode a repressor molecule that down-regulates levels of EPS production. Nonetheless, although other factors are invariably involved, it is suggested that EPS is a major compound that facilitates biofilm production and its level of expression ultimately determines the degree of biofilm formation.

Furthermore, it was an interesting observation that blotch discoloration of NZ103B5++ was slightly reduced (B7) in comparison to the wildtype NZ103 (B9). In a crude sense, this would suggest that EPS is not involved in blotch formation, however, one could also speculate that the overproduction of EPS may cause other factors to become involved in blotch. Firstly, EPS production is a metabolically expensive process and therefore the cell density may take longer to establish on the mushroom cube and/or energy may be diverted from other blotch causing processes. Secondly, EPS overproduction may form an external barrier for the cell that masks the exportation and/or contact of other compounds involved in blotch with *A. bisporus* tissue. Either way, it further confirms the complex nature likely to exist in the blotch processes and EPS, if involved, will only be a contributing part of the multifactorial process.

5.5.3 Hydrophobic EPS in attraction to surfaces / non suspension in ddH_2O

During the TEM analysis it was observed that the wildtype NZ103 and biofilm positive mutants (including 103B5++) were difficult to resuspend in water and formed coagulations of bacterial cells. In comparison, all biofilm negative mutants (103G3, D2, E2, F2, A5, and A3) resuspended into ddH_2O easily forming a uniform cell suspension with minimal physical agitation. This observation in conjunction with the TEM observations of a reduced EPS stained outer cell, suggests that biofilm deficient mutants have a reduced production of E*P*. As EPS consists of generally hydrophobic molecules, this could explain why NZ103 mutants in EPS production were easily suspending in

ddH_2O. In addition, this could also explain why these NZ103 mutants were unable to form biofilms on the hydrophobic PVC surface in that without the hydrophobic EPS, the mutant may encounter repulsion forces exerted by the hydrophobic PVC surface. If this was indeed the case, it would further suggest the involvement of EPS in biofilm formation and microscope studies should be employed to determine whether EPS mutants are involved in attachment and/or cluster development.

5.6 Motility analysis of biofilm defective strains

Large proportions of biofilm mutants described in previous studies have exhibited defects in flagella motility (O'Toole and Kolter, 1998b, O'Toole and Kolter, 1998a, Pratt and Kolter, 1998, Watnick and Kolter, 1999). However, this study showed that mutants unable to form biofilms, and those with a reduced biofilm phenotype, had comparable flagella synthesis and motility that of the NZ103 wildtype. The observation that motility and biofilm formation are not functionally linked is not unique and has been reported previously (Robleto, *et al.*, 2003). Although these results cannot exclude flagella mediated motility being involved in biofilm formation, this observation in NZ103 strongly suggests that it is not a critical factor in all environments. To further test the requirement for flagella motility in biofilm formation, motility defective mutants were isolated from the random pool of mutants and screened in the *in vitro* biofilm assay with all of these mutants demonstrating wildtype levels of biofilm formation (Table 5-1). TEM analysis of the motile NZ103 wildtype showed multiple polar flagella and small pili like structures. In contrast, the non-motile mini-Tn*5*km*lac*Z2 mutants 103G21, D24, B26, D25, and C29 all had no flagella visible. They did have the 'pili-like' structure remaining that were typically located in the polar region where the wildtype flagella would be. The remaining motility impaired mutants all had flagella indistinguishable to NZ103 wildtype. It is most likely that the mini-Tn*5*km*lac*Z2 insertion has disrupted genetic regulation of genes involved in the movement of flagella, not the structural genes. Nonetheless, these results further confirm that flagella are not a critical factor in the biofilm formation of NZ103, but are still likely to be critical in motility and chemotaxis toward *A. bisporus* hyphae (Rainey, 1989). It is likely that given the defined parameters of the abiotic biofilm assay that attachment to the PVC surface can occur without motility and furthermore that because this is a static environment, cells will remain attached even without flagella. Further investigation is needed to show whether flagella are required for *in situ* attachment of NZ103 on *A. bisporus*.

5.7 Correlation between biofilms and blotch

Both biofilms and blotch are likely to involve the coordinated expression of multiple genes in response to environmental signals. The suggestion that common global regulatory pathways might exist for both phenomena was further supported by the observation of all but one biofilm defective mutants (103G4) exhibited reduced blotch (B5 or less) in mushroom tissue assays compared to NZ103

wild type (B9). Of these biofilm mutants showing a reduced blotch phenotype, 103G2, 103G3, and 103G4 (Table 5-1) were also characterised as having reduced growth in M9 supplemented with glucose. However, when 103G3 growth rate in LB was compared to that of the wildtype, it was shown to be as effective, if not better (Figure 5-6). With the exception of these three mutants, the remaining biofilm mutants deficient in blotch formation showed growth patterns not visually different to that of the wild type suggesting that their mutations were having a more direct effect on the ability to form biofilms and blotch.

5.8 Analysis of growth rates of mutants in comparison to the wildtype

It has been argued that if a bacterial function contributes to the fitness of the organism *in vivo*, it may be defined as a virulence factor given that the evolution of virulence is subject to effective growth on host tissue and subsequent transmission to new hosts (Heithoff, *et al.*, 1997). Therefore, results of growth rates on nutrient depleted media such as M9 and MJA yielded interesting results.

5.8.1 Mutants deficient in the ability to grow on M9

In this study, the ability to grow on M9 medium, a medium that requires an organism to have prototrophic growth characteristics (*i.e.* have no nutritional requirements other than a carbon, water, and mineral source) was used to test the fitness of NZ103 mutants. NZ103 wildtype bacteria are prototrophic, and therefore any mutants unable to grow, or showing reduced growth on M9, were suggestive of insertional mutations within gene(s) involved in the synthesis of cellular processes required for prototrophic growth.

Of the mutants unable to grow as efficiently as the wildtype NZ103 (Table 5-1) nine exhibited no growth on M9, and four showed reduced growth in comparison to the NZ103 wildtype. An interesting observation was that with exception of 103E10, mutants that were unable to grow on M9 were able to grow on MJA and LB agar. This would suggest that these mutants had insertion sites within genes responsible for synthesis of many prototrophic requirements that are supplied both in MJA and LB.

When comparing mutants only exhibiting M9 altered phenotypes, it was interesting to observe a range of blotch phenotypes, as examples: NZG10 gave B8; 103E15 gave B7; whereas 103F3 gave B3 and G11 gave B4. Given that all these mutants were able to grow on MJA, it would suggest that the mushroom tissue provides sufficient nutrients for bacterial growth of these mutants and that blotch discolourations may be partly due to the genes involved in prototrophy, but the variation of blotch discolorations among these mutants is not a consequence of auxotrophy *per se*.

5.9 MJA growth

MJA was developed in this study to provide a medium that contained only those nutrients provided by the mushroom sporophore. This was deemed an important screen for NZ103 mutants, as the ability

to successfully compete and multiply given only the nutrients supplied in a particular niche is essential for pathogenicity. Results showed a number of NZ103 mutants had an altered ability to grow on MJA (Table 5-1) when compared with NZ103 wildtype growth. One isolate (103F5, discussed below) was unable to grow on MJA, and 11 mutants showed reduced growth compared to wildtype (103A1, B1, D1, E1, D2, E2, F2, G2, G3, D4 and D24). When selected MJA altered mutants growth rates in LB broth culture were analysed compared with NZ103 wildtype, it was shown that 103F2 and 103G3 grew equally well whereas NZ103F5 grew at ca. 70% efficiency. Furthermore, with exception of 103G2 and 103G3, although reduced growth on MJA was observed, growth on M9 media remained at wildtype levels (in respect to colony size relative to time). These observations combined, would indicate that MJA altered mutants remain prototrophic, but the transposon insertion had disrupted genes that enable efficient utilisation of MJA nutrients. This would suggest that the difference in M9 and MJA represents why these mutants are unable to efficiently grow. A plausible explanation may be the salt content in MJA is lower than M9 and the mutated gene is involved in osmotic regulation and cell viability.

All mutants exhibiting reduced growth on MJA could also be associated with the loss of at least one other putative pathogenicity determinant. This suggests that the mutation either; (i) has had polar effects on the expression of downstream genes; or (ii) has occurred within a global regulator of multiple phenotypes. Furthermore, with exception of 103D4, all MJA reduced isolates also showed deficiency in the ability to form wildtype blotch discolourations. This would suggest that these mutants are unable to efficiently grow on nutrients provided by the mushroom sporophore and therefore the reduction in blotch may not signal a reduction in a PPD(s) but rather, an inability to multiply to a cell-density threshold required for blotch discolouration.

5.9.1 NZ103 mutant 103F5 inability to grow on MJA only

Of special note was the isolate, 103F5, that was completely unable to grow on MJA and this mutant generated special interest as no other phenotypic deficiencies were observed using the assays used in this study. This suggested that the mini-Tn*5*km*lac*Z2 insertion was within a gene that was directly or indirectly involved with the ability of NZ103 to grow on nutrients extracted from sporophore tissue. 103F5 was also observed to cause a significantly reduced blotch phenotype in the mushroom cube bioassay, presumably because 103F5 cannot grow on MJA, and would therefore, also be unlikely to grow on mushroom tissue itself. This single mutation further shows that initial colonisation and efficient growth is the primary factor essential to pathogenicity. 103F5 was chosen for further genetic characterisation in Chapter 6 as elucidation of such genes would further the understanding of the molecular mechanisms occurring between BCOs/*A. bisporus*.

5.10 Mutants deficient in production of extracellular compounds

5.10.1 Protease and lipase production

Many mutants were recovered with a phenotype that was deficient in the production of either protease and/or lipase production. Mutants deficient in both tended to also be deficient in other phenotypes such as biofilm formation and reduction of growth on M9 (Table 5-1); which is suggestive that either: (i) these genes are spatially linked on the NZ103 chromosome and the mini-Tn*5*km*lac*Z2 insertion has caused a polar mutation; or (ii) insertion has occurred within a global regulator of both enzymes production and/or extracellular export.

It is of interest to look at the single mutant phenotype deficiencies and compare them to their ability to induce blotch. Mutants deficient in only protease production, and not in any other of the phenotypes tested, included 103C13 and 103E3. They were both observed to cause blotch discolourations of B6. It is tempting to suggest that protease production is therefore, not the factor that is responsible for wildtype blotch discolouration (B9), however, there is a reduction in blotch and it is has to be assumed that other pathogenicity determinants not tested in this study have been affected by the genetic insertion of mini-Tn*5*km*lac*Z2 in these mutants. This is further demonstrated with analysis of 103A13 and 103D13, both deficient in only lipase production, were inconclusive as to the involvement of lipase in blotch formation as they produced B7 and B4 blotch discolourations respectively. Nonetheless, the development of such PPD assays should be viewed as providing more than a positive/negative approach to pathogenicity. These assays have identified important factors that are involved in the reduction of a given phenotype, and should be viewed as a tool of identifying components within the complex mechanisms of bacterial interactions.

5.11 EPS involvement in biofilm formation

There was initial observation that colony morphology was altered with NZ103 mutants unable to form biofilms. Morphology was less mucoidy within the primary streak, although the growth rates were compared to NZ103 wildtype. When the outer cell wall was visualised by TEM, further differences were observed between biofilm mutants and biofilm positive NZ103 strains. As discussed previously, the dark staining around bacterial cells under TEM was considered a result of the outer cellular matrix that contains proteins, polysaccharides and other such molecules (Rainey, 1991). It is apparent in the observation of the three strains 103G3 (biofilm negative), NZ103 wildtype (biofilm positive) and 103B5++ (biofilm enhanced) that the degree of this outer staining is variable, with the least produced in the 103G3 and the most in 103B5++ (Figure 5-7). This suggests that the dark stained extracellular polysaccharides are likely to have an important role in biofilm formation.

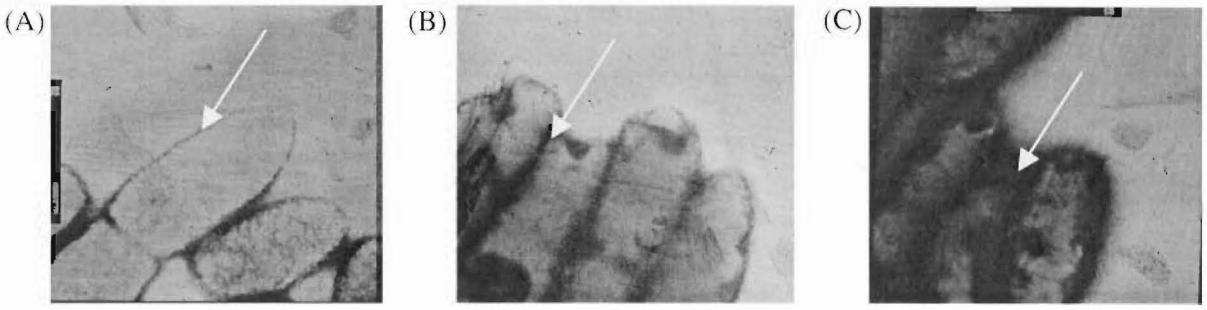


Figure 5-7 TEM comparison of the (A) biofilm deficient mutant 103G3, (B) biofilm positive NZ103 wildtype and (C) biofilm enhanced 103B5++. Arrows indicate the increase of the dark stained extracellular polysaccharides.

It was also observed under TEM that biofilm negative mutant cells were not aggregated together as in the 'clumped' nature observed in biofilm positive NZ103 mutants. This would further suggest that the EPS is required for cell-to-cell adherence. However, it should be noted that the synthesis and excretion of bacterial EPS is an energy intensive process and consequently EPS production is likely to be under strict regulatory control (Costerton, 1985). The observation of mutants that appear to exhibit both decreases (e.g. 103G3) and increases (103B5++) in EPS production indicates that there are regulatory mechanisms in NZ103 for both up- and down-regulation of an EPS structural genes. This would suggest that the mini-Tn*5*km*lacZ*2 insertion into the biofilm deficient mutants may have interrupted a regulatory gene responsible for the up-regulation of EP. Likewise, and the mini-Tn*5*km*lacZ*2 may have inserted in 103B5++ interrupting a regulatory gene involved in the down-regulation of EPS synthesis. The gene for up-regulation of EPS is likely to be a regulatory protein that has multiple functions and would explain why other phenotypes have been affected in these biofilm mutants. Such mutants made suitable candidates for genetic characterisation in the following chapter.

5.12 Selection of specific Tn5 mutants to undergo genetic characterisation

The results of the phenotypic characterisation of NZ103 mutants with the aforementioned assays resulted in the identification of many NZ103 mutants deficient in one or a combination of the putative pathogenicity determinant phenotypes (Table 5-1). Twenty-one of these mutants defective in PPDs were further shown to also have a marked effect on the ability of mutants to induce blotch in mushroom tissue bioassay. From these 21, 10 mutants were chosen (Table 5-2) for genotypic analysis in the following chapter.

Table 5-2 Summary of the ten NZ103 mutant phenotypes selected for further characterisation. Mutants are categorised with respect to their respective phenotypes.

Mutant	Biofilm	Lipase	Protease	M9	Mush J	Motility	Blotch Scale
103 WT	+	+	+	+	+	+	B9
103 G3	-	-	-	R	R	+	B1
103 F3	+	+	+	-	+	+	B3
103 A5	-	+	-	+	+	+	B2
103 F5	+	+	+	+	-	+	B3
103 A14	+	-	-	+	+	+	B3
103 D13	+	-	+	+	+	+	B4
103 A3	-	-	-	+	+	+	B2
103 D2	-	-	-	+	R	+	B3
103 E2	-	-	-	+	R	+	B1
103 F2	-	-	-	+	R	+	B2

- Does not exhibit the selected phenotype
+ exhibits the phenotype comparable to wild type NZ103
R growth is reduced compared to wildtype NZ103

These ten mutants were selected because they (i) exhibited substantial reduction in blotch formation, and (ii) represented diversity in their deficiency of putative pathogenicity determinant phenotypes. Because these ten NZ103 mutants exhibited reduced blotch activity, they were termed BCO^R mutants[1]. The genetic characterisation of the mini-Tn*5*km*lacZ*2 insertion point in these ten NZ103 mutants is the focus of the following chapter.

[1] Where the 'R' in BCO^R signifies 'reduced'.

CONCLUSIONS

This section of study was initiated to identify and characterise selected genes involved in blotch formation by NZ103. The scope of this study was limited to the screening of only 5000 mutations generated by the same mutagenic element, mini-Tn*5*km*lac*Z2 within defined assay conditions to determine loss of specified pathogenicity determinants. As described previously, it is acknowledged that the types of NZ103 mutants isolated during this study were undoubtedly influenced by the conditions of the assay conditions chosen. When considering the multi-factorial aspect of pathogenesis, it is unrealistic to expect to develop methodologies that encompass elucidation of all aspects of pathogenicity of a selected organism. Therefore, results presented in this thesis are likely to represent only a percentage of the actual number of genes involved in the blotch formation of NZ103. A more thorough mutagenesis screen using different mutagenic agents, screening conditions, and a greater number of mutants may well gain a better understanding of the range of genes involved in blotch formation. However, given that the time frame of this study did not allow for such an approach, it was considered that the ten NZ103 mutants selected (Table 5-2) for genetic analysis in the following chapter would provide insight into critical factors involved in the blotch discolouration process.

Chapter 6

GENETIC CHARACTERISATION OF SELECTED TN5 NZ103 MUTANTS WITH A REDUCED BLOTCH PHENOTYPE

Ten NZ103 mutants were selected (Chapter 5) based on the loss of one or a number of PPDs and a reduced ability to cause blotch discolouration of *A. bisporus* tissue (BCO^R). The assumption has been made that the transposon has disrupted a gene(s) that is responsible for the expression of that given phenotype. Furthermore, that the insertion site may be either: (i) a gene directly responsible for the expression of the particular phenotype; (ii) this insertion has caused a polar effect on adjacent genes thus eliminating correct expression of the phenotype; or (iii) insertion may have occurred in a global regulator gene that is responsible for the expression of many phenotypes. This chapter presents sequence data relating to the transposon insertions of the ten BCO^R mutants and discusses similarity to previously described genes and whether such disruptions correlate with the altered phenotype(s).

6.1 Objectives for Chapter 6

Based on the rationale outlined above, the ten selected mutants (Table 5-2) were used to address the following objectives:

1. Sequence DNA flanking the mini-Tn*5*km*lac*Z2 from the selected NZ103 mutants.
2. Compare NZ103 nucleotide sequence to existing databases and where possible, assign nucleotide sequence and translated protein similarities to deposited sequences.
3. Correlate putative gene disruptions with individual mutant phenotypes with an aim to gaining insight into the types of gene(s) and processes involved in bacterial/fungal interactions and blotch disease of *A. bisporus*.

RESULTS AND DISCUSSION

All commonly used methodologies in this section, including DNA manipulation, plasmid extractions, restriction map analysis, cloning and nucleotide sequencing are described in Appendix I.

6.2 *lacZ2* fusion status

For the purpose of general characterisation, the *lacZ* translational fusion status of the NZ103 mutants was assessed. The mini-Tn*5*km*lacZ*2 contains a *lacZ* gene that lacks transcriptional or translational signals, and can therefore be used to create protein fusions. Such fusions can be utilised to provide quantitative information on the interrupted gene(s) translation, but more importantly, and active translational fusion indicates that an actively translated coding region has been disrupted. NZ103 mutants were assessed for active translational fusions by plating colonies onto LBA supplemented with 5-bromo-4-chloro-3-indoyl-b-galactopyranoside (Xgal). Xgal is a chromogenic substrate that produces a blue compound when cleaved by β-galactosidase. No β-galactosidase activity was observed for the BCO^R mutants tested from Table 5-2 over a 72 hr period, which suggest the mini-Tn*5*km*lacZ*2 did not create a protein fusion within these mutants.

6.3 Cloning of mini-Tn*5*km*lacZ*2 NZ103 insertion sites

Sequence data from DNA flanking the transposon was obtained for all *P. putida* NZ103 mutants listed in Table 5-2. Genomic DNA fragments containing transposon insertions were initially cloned into pBluescript KS- (pSK-) (Stratagene, Appendix IV). NZ103 DNA fragments were generated by separate restriction digestions with restriction enzyme *Pst*I[1] as neither of these enzymes cut within the transposon (Figure 6-1).

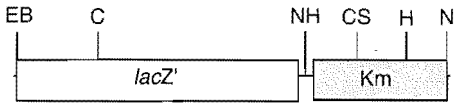


Figure 6-1 Schematic diagram of mini-Tn*5*km*lacZ*2 (de Lorenzo *et. al.*, 1990). Shown are the restriction sites, genes and designated left and right ends. Abbreviations for restriction endonucleases: E, *Eco*RI; B, *Bam*HI ; C, *Cla*I ; N, *Not*I; H, *Hind*III; S, *Sma*I.

[1] Note that SalI is another enzyme that does not cut in mini-Tn*5*km*lacZ*2 and was used in this study, however all resulting KmR clones resulted from the *Pst*I enzyme.

Digested DNA, was ligated into complementarily restriction digested pSK- vector and colonies were recovered on LB agar supplemented with Ap (for selection of pSK-) and Km (for selection of mini-Tn*5*km*lacZ*2). Resulting colonies were recultured on selective media and plasmids were purified from isolates exhibiting stable antibiotic resistance. Restriction map analysis was performed using enzymes that do not cut within mini-Tn*5*km*lacZ*2 and any plasmids exhibiting identical restriction maps from the same restriction digested NZ103 mutant were assumed to be clonal and only one was arbitrarily chosen for further analysis. A single recombinant plasmid containing a unique restriction map was selected for each NZ103 mutant from DNA sequence analysis using the following sequencing strategies.

Determination of NZ103 flanking DNA either side of mini-Tn*5*km*lacZ*2

For each individual NZ103 DNA clone from the ten NZ103 mutants (Table 5-2), further restriction maps were obtained to determine placement of mini-Tn*5*km*lacZ*2 within the cloned NZ103 DNA fragment. This information is schematically represented under each mutant clone analysis. The total size of the NZ103 DNA fragment was determined using the enzyme originally used to clone the mini-Tn*5*km*lacZ*2 DNA fragment into pSK-. As the pSK- vector is 3.0 kb, any secondary DNA band visualised on the agarose gel was assumed to be mini-Tn*5*km*lacZ*2 flanked by NZ103 DNA. As mini-Tn*5*km*lacZ*2 is 5.2 kb the amount of total flanking NZ103 DNA was calculated accordingly. Further restriction mapping was performed using enzymes that do not cut within mini-Tn*5*km*lacZ*2 to determine the size of NZ103 DNA fragments flanking either end the mini-Tn*5*km*lacZ*2. It was this size that ultimately determined which of the following nucleotide sequencing strategies was used.

6.3.2 Sequencing strategies of DNA flanking mini-Tn*5*km*lacZ*2

Sequencing strategies were developed to obtain NZ103 nucleotide sequence flanking the mini-Tn*5*km*lacZ*2 insertion site from pSK- clones obtained in section 6.3. Information pertaining to the insertion site of mini-Tn*5*km*lacZ*2 within the NZ103 genome was sought by obtaining nucleotide sequence that spanned the Tn*5*/chromosome junction site. To obtain such information, different cloning strategies had to be utilized depending on the size of the cloned NZ103 DNA fragment either side of the mini-Tn*5*km*lacZ*2. Realistically, only ca. 800bp of informative nucleotide sequence is expected from current sequencing protocols. Therefore, strategies were devised for sequence fragments no greater than 750 bp so that nucleotide sequence information would include the junction site of mini-Tn*5*km*lacZ*2 in NZ103 genomic DNA.

6.3.3 pSK- containing insert DNA <750 bp either side of mini-Tn*5*km*lacZ*2

If the desired DNA fragment located between the mini-Tn*5*km*lacZ*2 insertional point and pSK- clone site was less than ca. 750 bp, sequencing was carried out directly from pSK- using the respective incorporated oligonucleotide primer site (either T7 and T3) (Figure 6-2).

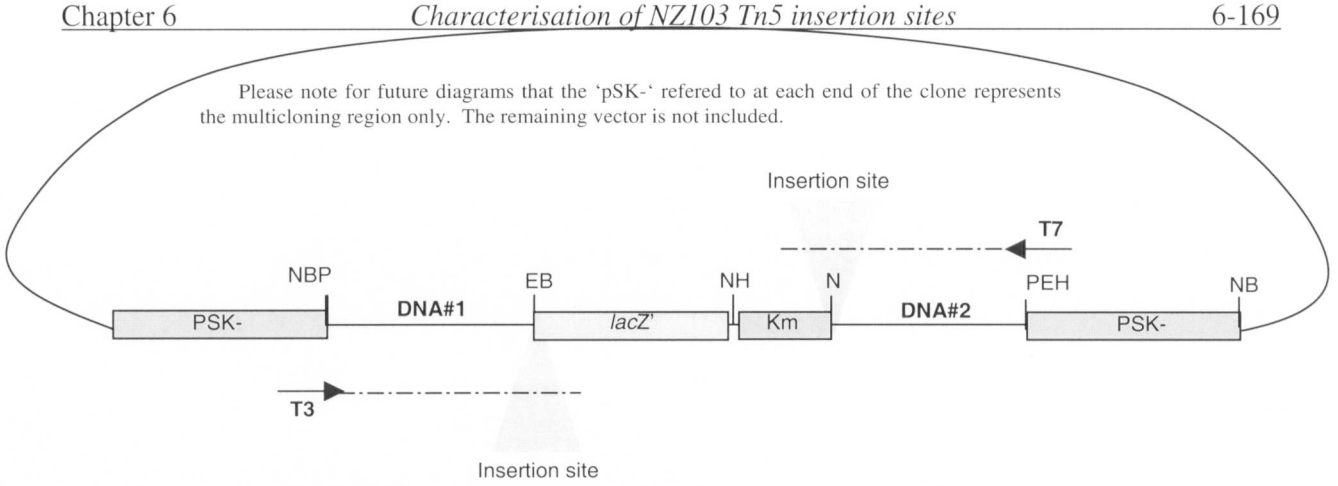


Figure 6-2 Sequencing pSK- with T3 and T7 oligonucleotide priming sites provided either side of the pSK- multicloning site (Stratagene). This strategy was used in this study if DNA#1 and/or DNA#2 was less than ca. 750bp and therefore, allowed sequence information of the mini-Tn*5*km*lac*Z2 insertion site within NZ103 genomic DNA.

6.3.4 pSK- containing insert DNA >750 bp either side of the transposon

pSK- clones with NZ103 DNA sequence(s) flanking mini-Tn*5*km*lac*Z2 that were of a length greater than ca. 750 bp were subcloned for sequencing purposes using one of following two methods.

(i) Subcloning smaller fragments back into pSK-

If the desired NZ103 DNA fragment contained appropriate enzyme restriction sites, it was digested and subcloned back into the appropriately digested pSK- as depicted in Figure 6-3.

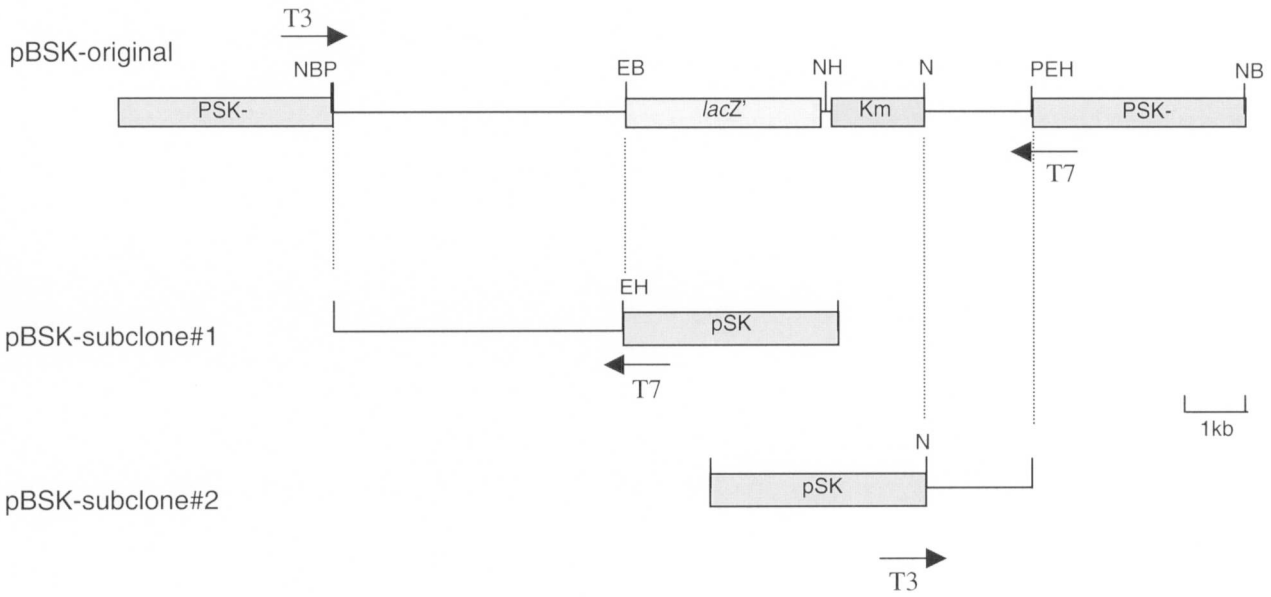


Figure 6-3 Schematic representation of subcloning of NZ103 DNA fragments greater than 750 bp from pSK- into pSK- to allow sequencing of insertion region by either T3 or T7.

This strategy was based around the utilisation of the unique *Eco*RI site at the left end of the transposon and the *Not*I site at the right end of mini-Tn*5*km*lac*Z2 (Figure 6-1). Digestion with these enzymes, and cloning back into pSK- enabled the resulting DNA fragments of appropriate size to again be sequenced with either T3 or T7 primers (Figure 6-2). Both the *Eco*RI and *Not*I restriction sites are at the ends of mini-Tn*5*km*lac*Z2 and therefore subclones could still provide information as to the mini-Tn*5*km*lac*Z2 junction site in NZ103 DNA[1].

(ii) Shotgun cloning of the total fragment from pSK- into pBR322

This second strategy was used for flanking DNA that could not be subcloned back into pSK- because of a lack of appropriate enzyme sites. Essentially, the total NZ103 DNA fragment containing mini-Tn*5*km*lac*Z2 was digested from pSK- with the enzyme used to originally clone the fragment and was then shotgun cloned (Appendix I) into pBR322 (New England Biolabs) as depicted in Figure 6-4.

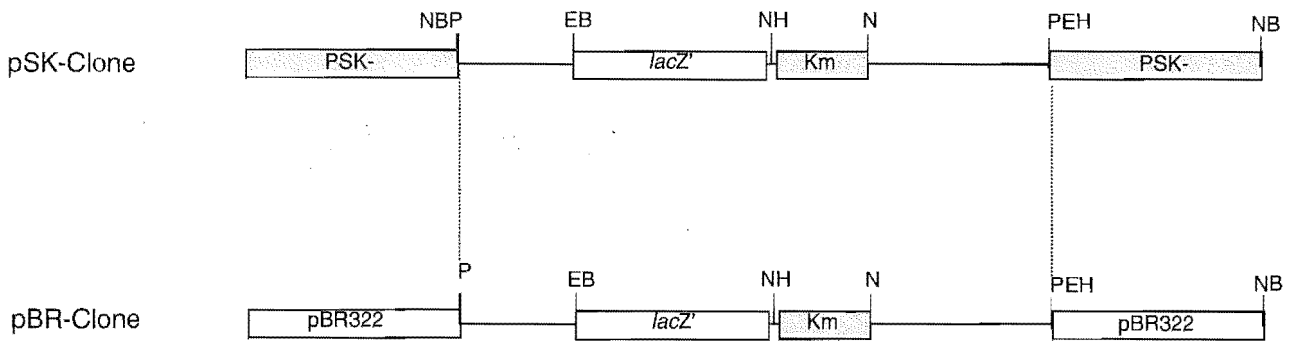


Figure 6-4 Schematic representation of subcloning of total NZ103 DNA fragment containing mini-Tn*5*km*lac*Z2 from pBluescript SK- into pBR322.

Selection of pBR322 subclones containing the DNA fragment was achieved by selection of colonies on LBA supplemented with Tc (to select for pBR322 and counter-select against pSK-) and Km (to select for mini-Tn*5*km*lac*Z2). Colonies were screened by restriction mapping as described above to ensure efficiency of subcloning procedures.

This strategy was used in order to utilize the transposon sequence as oligonucleotide primer binding sites in combination with primer sites within pBR322 to generate PCR amplicons (Figure 6-5). These PCR amplicons could then be used to obtain direct nucleotide sequence information of the DNA either side of mini-Tn*5*km*lac*Z2 using the appropriate oligonucleotide sequencing primer[2].

---

[1] Note that this was not always achievable in circumstances where either *Eco*RI and/or *Not*I was present within the NZ103 genomic fragment.

[2] Primer sequences are presented in Appendix VI.

Nucleotide sequencing using either Tn*5*-O or lac-no.18 oligonucleotide primers enabled the mini-Tn*5*km*lac*Z2 insertion site to be determined for clones with transposon flanking regions under 5kb.

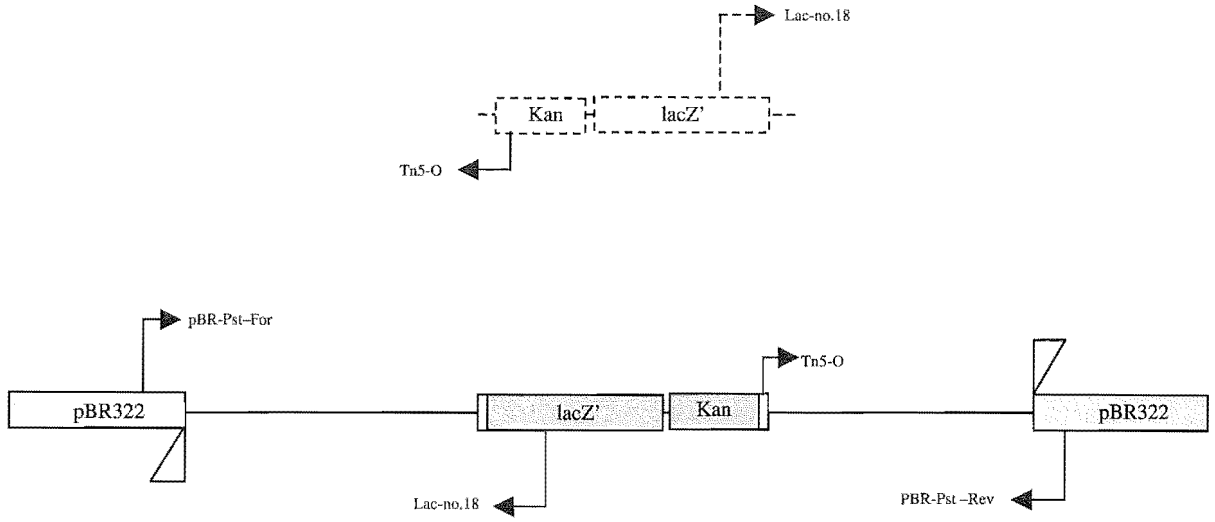


Figure 6-5 Oligonucleotide primer amplification of DNA fragments flanking mini-Tn*5*km*lac*Z2 contained within pBR322. Primers pBR-Pst-Rev, PBR-Pst-Fwd, Tn*5*-O and lac-no.18 were designed in this study and utilized to obtain sequence information of the mini-Tn*5*km*lac*Z2 insertional site within NZ103 genomic DNA.

The reason this strategy of using oligonucleotides primed off the end of mini-Tn*5*km*lac*Z2 could not be employed in pSK- is both mini-Tn*5*km*lac*Z2 (Simon, 1989) and pSK- contain the *lac*Z gene (Stratagene). Therefore, the use of oligonucleotide primers designed for the *lac*Z region of Tn*5* would also prime from the *lac*Z in pSK- and give non-sense nucleotide sequence information from primer amplifications defined in Figure 6-5. However, pBR322 does not contain the *lac*Z gene and therefore to use this PCR amplification strategy genomic DNA fragments were required to be cloned with pBR322.

PRELIMINARY SEQUENCE DATA ANALYSIS

In the following sections, genotypic analysis of the mini-Tn*5*km*lac*Z2 insertion sites within the ten selected NZ103 mutants (Table 5-2) is presented. Initially, restriction profiles are depicted in schematic maps for individual genomic clones to show the size of DNA flanking mini-Tn*5*km*lac*Z2. Using the appropriate aforementioned sequencing strategy, nucleotide sequence was determined for each mini-Tn*5*km*lac*Z2 insertion site and deposited to GenBank under the accession numbers presented in Table 6-2. Information is provided as to the amount of nucleotide sequence obtained, GenBank accession numbers of reported matches from submissions to the BlastX and BlastN server (www.ncbi.nlm.nih.gov/blast). Note that accession numbers and statistical significance of these referenced matches from GenBank are presented in a summary table at the completion of this section.

In the following sections, the closest GenBank match is not always mentioned where identity is to a 'putative' or 'hypothetical' protein with unassigned function from genome sequencing projects. Only similarities to genes from previous bacterial studies that have assigned functionality are presented[1]. Therefore, GenBank identities have been used as an initial means to gaining insight into the nature of NZ103 BCO^R mutations. In concert with the significant amount of phenotypic information accumulated for each mutant (Chapter 5), sequence data was hoped to provide identification of specific molecular pathways involved in bacterial/fungal interactions and/or blotch discolourations.

6.3.1 p103G3 (Bfm$^-$, Lip$^-$, Prot$^-$, M9^R, MJA^R, Mot$^+$, B1)

Characterisation of clone

An ca. 7.2 kb *Pst*I fragment containing mini-Tn*5*km*lac*Z2 was cloned from NZ103-G3 into pBluescript KS- to generate the plasmid pBSK-G3-2. Restriction analysis of the clone pBSK-G3-2 indicated that 850 bp and 1.5 kb of genomic DNA was flanking the Km and *lac*Z ends of the transposon respectively (Figure 6-6).

[1] Note that if a publication has resulted from matched sequence, it is cited, however, not all submissions have associated publications at time of sequence analysis.

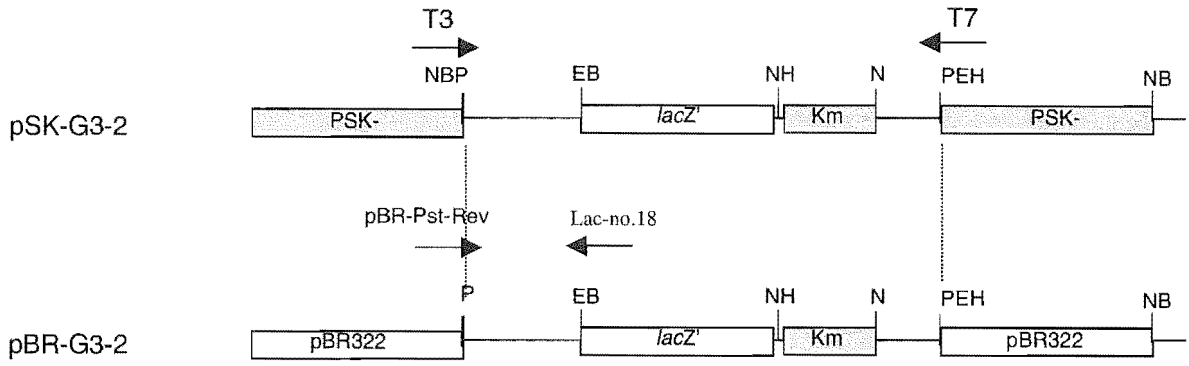


Figure 6-6a Schematic diagram of the clone and subclone derived from NZ103 DNA flanking the mini-Tn*5*km*lacZ*2 insertion point in p103G3. Dashed lines indicate the fragment that was subcloned. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ*2

Inserts were sequenced with T3 and T7 from either side of the pSK- cloning site. Approximately 750 bp and ca. 950 bp of sequence information was obtained respectively. As the T7 sequence was not sufficient to reach the insert site at the Km end of the transposon, shotgun cloning was performed to move the *Pst*I fragment containing mini-Tn*5*km*lacZ*2 into pBR322. This gave the subclone pBR-G3-2. Further nucleotide sequence was obtained from the transposon insertion site by generating a 1.5 kb PCR amplicon with oligonucleotides PBR-Pst-Rev and lac-no.18 (Figure 6-6a). This was direct sequenced (Appendix I) using the lac-no.18 primer and provide ca. 1000 bp of sequence. The three sequences were combined and a consensus sequence was derived and deposited in GenBank (AY224670).

Sequence comparisons to GenBank

When the consensus nucleotide sequence from 103G3 was compared to submissions contained in GenBank, similarity was shown at the nucleotide level within a *P. tolaasii* gene for sensor kinase *rtpA* (Murata, *et al.*, 1998), *P. stutzeri* JM300 *gacS*, and the *P. fluorescens* Pf-5 *adpA* (Corbell and Loper, 1995). Furthermore, translation prediction gave similarity to *Pseudomonas chlororaphis* GacS (Chancey, *et al.*, 2002) as well as *P. tolaasii* RtpA (Murata, *et al.*, 1998). The *gacS* and *rtpA* genes have been identified as having functional involvement in two-component regulatory systems.

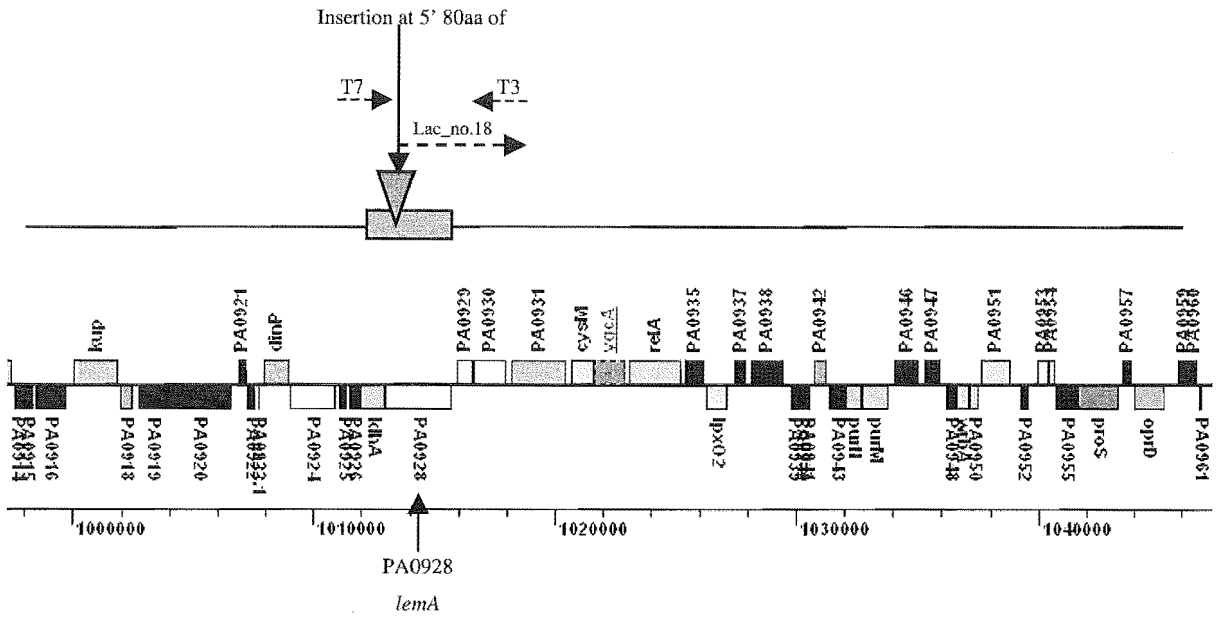


Figure 6-6b Insertional site of NZ103-G3 given protein translational comparison with *P. aeruginosa* PAO1. Dashed arrows indicate generation of sequence

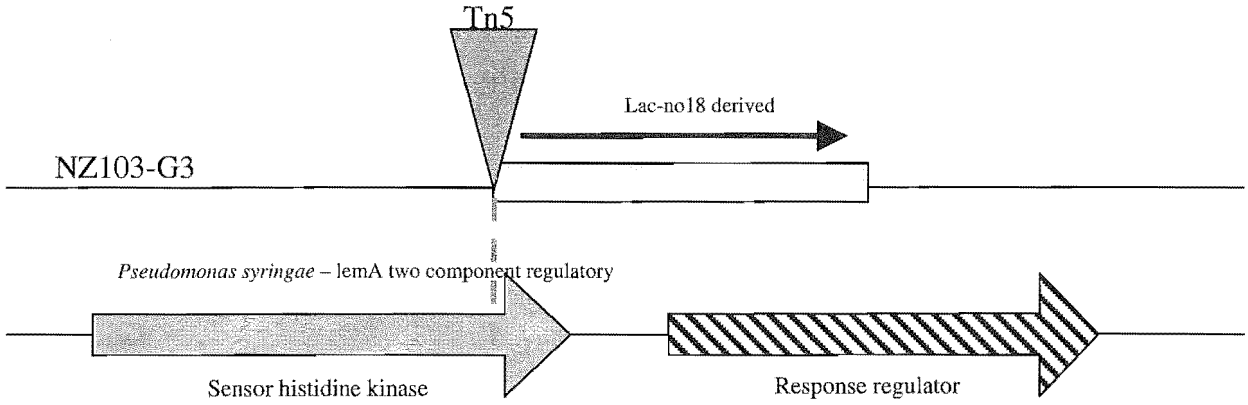


Figure 6-6c Relative position and placement of NZ103-G3 Tn*5* within the Sensor histidine kinase of lemA from *P. syringae*

Two component regulatory systems

Signals from within the cytoplasm, and from the environment, activate bacterial adaptive responses ranging from rapid transient changes in motility to long-term global reorganisations of gene expression. Many pathogenic bacteria have phenotypes that are controlled by signals from the host or environment and regulatory elements in the bacterium respond to such stimuli (Dziejman and

Mekalanos, 1995, Alfano and Collmer, 1996). A prominent example of these are the bacterial two-component regulators (Stock, *et al.*, 2000). Two-component systems are widely distributed amongst bacteria and are believed to help adaptation to different conditions and to colonise specific ecological niches in response to environmental signals (Heeb and Haas, 2001). Typically, a two-component system consists of a sensor kinase and a cognate response regulator. Upon interaction with the signal(s), the sensor undergoes autophosphorylation and then activates the response regulator by phosphotransfer. The signals may be abiotic (e.g. pH, temperature, osmolarity) or biotic; some signals are produced by a host, others synthesised by the bacterial populations[1] themselves (Stock, *et al.*, 2000) but, for a number of two-component systems, the signals are not yet known.

***P. tolaasii* rtpA**

In a previous study, *P. tolaasii* strain PT814 was mutated with transposon mini-Tn*5*km1 to select for pleiotropically defective mutants in tolaasin, protease production, or altered in colony morphology (Murata, *et al.*, 1998). Within this study, the gene, *rtpA* was found to be involved in the regulation of tolaasin production and other pleiotropic traits. Nucleotide sequencing of *rtpA* revealed a 2.75 kb ORF predicted to encode a protein (917 amino acids) displaying the conserved amino acid sequence of both sensor and receiver domains of "bacterial two-component regulators". This suggested that *rtpA* was involved in responding to environmental stimuli essential for the pathogenic interactions of *P. tolaasii* with the mushroom. Amongst fluorescent pseudomonads producing antifungal agents, two families have been identified; the *bvgS* and *gacA* families (Hrabak and Willis, 1992, Laville, *et al.*, 1992, Corbell and Loper, 1995). The *pheN* gene[1] of *P. tolaasii* strain NCPPB1116 required for tolaasin production (Han, *et al.*, 1997) is reported to belong to the *bvgS/lemA* family (Grewal, *et al.*, 1995). Regulators belonging to *bvgS* family include sensor histidine protein kinases in which signals are transmitted by a series of phosphorylation relays to the response regulator (Uhl and Miller, 1995). By contrast *gacA* is a response regulator which receives signals by phosphorylation and activates the expression of the target gene (Laville, *et al.*, 1992).

gacS* was formerly known as *lemA

The *gacS* gene was first described in phytopathogenic *P. syringae* pv. *syringae* as *lemA* (Hrabak and Willis, 1992). *P. syringae* pv. *syringae* isolate B728a is a causal agent of bacterial brown spot of bean (*Phaseolus vulgaris*) and during a Tn*5* screen of prototrophic mutants of B728a, a mutant was identified unable to form brown spot lesions on bean leaves and pods. However, the mutant retained the ability to colonise bean plants and elicit hypersensitive reaction on nonhosts (Willis, *et al.*, 1990). This mutation, defined as *lemA* (lesion manifestation) was also deficient in the ability to produce protease and the toxin syringomycin (Hrabak and Willis, 1990), suggesting that the *lemA* gene might encode a regulatory molecule. Evidence was further provided that the *lemA* gene had similarity to the

[1] For example, quorum-sensing as discussed in Chapter 4.3.3.

conserved domains within both the histidine kinase and the response regulator in a family of prokaryotic two-component regulatory proteins (Hrabak and Willis, 1992). This family of two-component regulatory proteins also include the VirA, BvgS, RcsC, and PhoR proteins (Leroux, *et al.*, 1987, Arico, *et al.*, 1989, Makino, *et al.*, 1989, Stout and Gottesman, 1990). Based on sequence similarities between identified *gacS* sequences, several functional domains have been postulated (Heeb and Haas, 2001) (Figure 6-7).

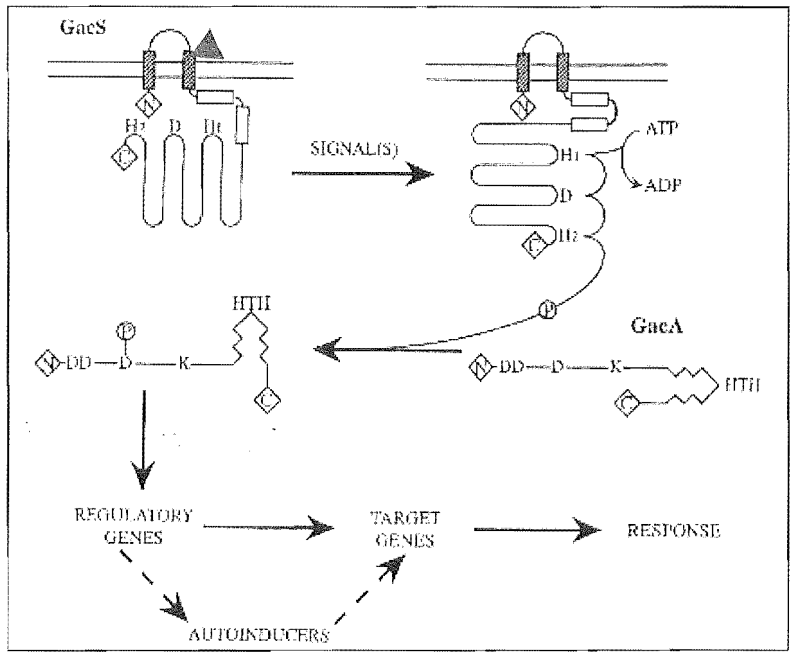


Figure 6-7 Model of the signal transduction pathway mediated by the GacS/GacA two-component system (Heeb and Haas, 2001). Autophosphorylation of GacS at the first conserved histidine residue (H1) is triggered by unknown signal(s). A phosphoryl group is transferred by a phosphorelay mechanism to conserved aspartate and histidine residues in the domains D and H2 respectively, of GacS. A conserved aspartate in GacA then acts as a phosphoryl acceptor. The phosphorylated response regulator GacA activates (or represses) their transcription of unidentified regulatory elements, which in turn control the expression of target genes, at least in part, at posttranscriptional level. In a few microorganisms, autoinducers (N-acyl homoserine lactones) are signals under GacS/GacA control. Red arrow indicates likely Tn5 insertion site in NZ103-G3 which would disrupt the senor histidine kinase region and therefore no environmental signals will get processed.

The more commonly used nomenclature for describing the gene with functions initially described as *lemA*, is now *gacS* (for global activator sensor kinase) (Whistler, *et al.*, 1998) and furthermore, *gacS* in other studies has different descriptions; such as *apdA* (Corbell and Loper, 1995), *repA* (Kitten, *et al.*, 1998), or *pheN* (Han, *et al.*, 1997).

[1] Previously discussed in Chapter 3.3.5.

GacS two-component regulatory systems

The sensor kinase, GacS, is an important member of a two-component regulatory system present in a wide variety of Gram-negative bacterial, including fluorescent pseudomonads (Heeb and Haas, 2001). The GacS/GacA systems controls the production of secondary metabolites and extracellular enzymes involved in pathogenicity to plants and animals, biocontrol of soil-borne plant diseases, ecological fitness, or tolerance to stress (Hirano and Upper, 2000, Heeb and Haas, 2001). The response regulator GacA was first described in *P. fluorescens* biocontrol strain CHAO as a global activator of antibiotic and cyanide production (Laville, *et al.*, 1992). Since publication of the initial reports, *gacS* and *gacA* homologs have been characterised in a great variety of other Gram-negative bacteria (Heeb and Haas, 2001) with the prominent common properties in mutants being either partial or complete loss in biocontrol ability, or markedly reduced virulence in plant- or animal-pathogenic bacteria (Liao, *et al.*, 1994, Corbell and Loper, 1995).

Similarity of 103G3 nucleotide sequence with D-lactate dehydrogenase *ldhA*

It was also observed that translation predictions of nucleotide sequence obtained from T7 from pSK-G3-2 gave high similarity to a D-lactate dehydrogenase gene *ldhA* from *P. putida* KT2440 (Nelson, *et al.*, 2002). This was further investigated using the annotated sequenced genome of *P. aeruginosa* PAO1 (http://www.*Pseudomonas*.com/index.html, (Stover, *et al.*, 2000)) and it was shown that the gene PA0928 (*lemA*) is located adjacent to *ldhA* in the genome (Figure 6-8).

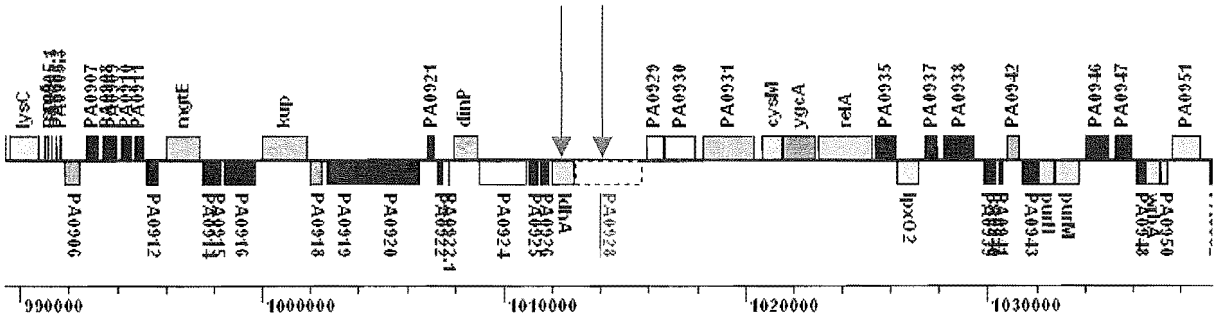


Figure 6-8 Proximal location of PA0928 (*lemA*) with *ldhA* in the *P. aeruginosa* PAO1 genome (http://www.*Pseudomonas*.com/).

Thus, *lemA, rtpA* and *gacS* are all descriptions of the same functional gene. This information suggests that the gene order within NZ103 is the same as *P. aeruginosa* PAO1 in that the *gacS/rtpA* homolog identified in p103G3 is adjacent to D-lactate dehydrogenase *ldha* within the chromosome.

Summary of p103G3

Mini-Tn5km*lacZ*2 insertion in 103G3 is likely to be in the sensor protein of a two-component system related to previously described genes *lemA/gacS/rtpA*. Furthermore, this two-component

regulatory protein in NZ103 appears to be required for the expression of a number of phenotypes, including biofilm formation, protease and lipase production, and effective growth on M9 and MJA[1].

6.3.2 p103F2 (Bfm$^-$, Lip$^-$, Prot$^-$, M9$^+$, MJA^R, Mot$^+$, B2)

Characterisation of NZ103 DNA insert clone

An ca. 10 kb *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was cloned from NZ103-F2 into pBluescript KS- to generate the plasmid pBSK-F2-1. Restriction analysis of pBSK-F2-1 indicated that ca. 5 kb and 100 bp of NZ103 DNA was present at the Km and *lacZ* ends of mini-Tn*5*km*lacZ*2 respectively (Figure 6-9).

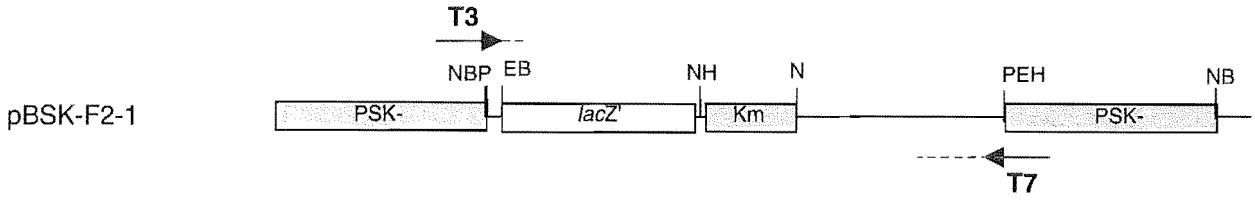


Figure 6-9 Schematic diagram of the clone of NZ103 DNA fragment containing the mini-Tn*5*km*lacZ*2 insertion site of p103F2. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ*2

Nucleotide sequence for DNA flanking the Km end of mini-Tn*5*km*lacZ*2 within pBSKF2-1 was generated using T7 to obtain ca. 940 bp sequence data (Genbank, AY224671) and T3 to obtain ca. 100 bp of NZ103 DNA sequence.

Sequence comparisons to GenBank

Although T7 nucleotide sequence did not include the mini-Tn*5*km*lacZ*2 junction site, it was still compared to GenBank. 103F2 nucleotide sequence (obtained from the T7 end) had predicted translation products with high similarities to the cysteine synthase B gene from many bacterial species, including *cysM* from *P. aeruginosa* PA01 (Stover, *et al.*, 2000), *P. putida* KT2440 (Nelson, *et al.*, 2002), *Vibrio cholerae*, *Salmonella enterica*, and *Azotobacter vinelandii*. The ca. 100 bp from T3 gave no similarity, probably because of its small size.

Spatial arrangement of 103G3 and 103F2 mini-Tn*5*km*lacZ*2 insertion sites

If the spatial location of the homologous gene to p103F2 of *cysM* (PA0932) from *P. aeruginosa* PAO1 is viewed (http://www.*Pseudomonas*.com/AnnotationByPAU.asp?PA=PA0932) it is found to be ca. 5 kb away from *gacS* (PA0928) homologue identified in 103G3. The close proximity of these

[1] Determined by phenotypic deficiencies reported within Table 5-2.

genes suggest that the actual insertion site of mini-Tn*5*km*lacZ*2 in 103F2 is within the *gacS* gene based on determination of sequence obtained from T7 and the size of the NZ103 DNA fragment flanking the transposon. The two mutants, p103G3 and p103F2, they have consistent mutant phenotypes (*i.e.* Bfm$^-$, Lip$^-$, Pro$^-$, MJA^R and BCO^R), suggesting that these two mini-Tn*5*km*lacZ*2 insertion sites may be spatially related within the *gacS* gene. This assumes the genetic order in NZ103 is the same as that described for *P. aeruginosa* PAO1 (Stover, *et al.*, 2000) in this region. More sequence information is required to validate this statement.

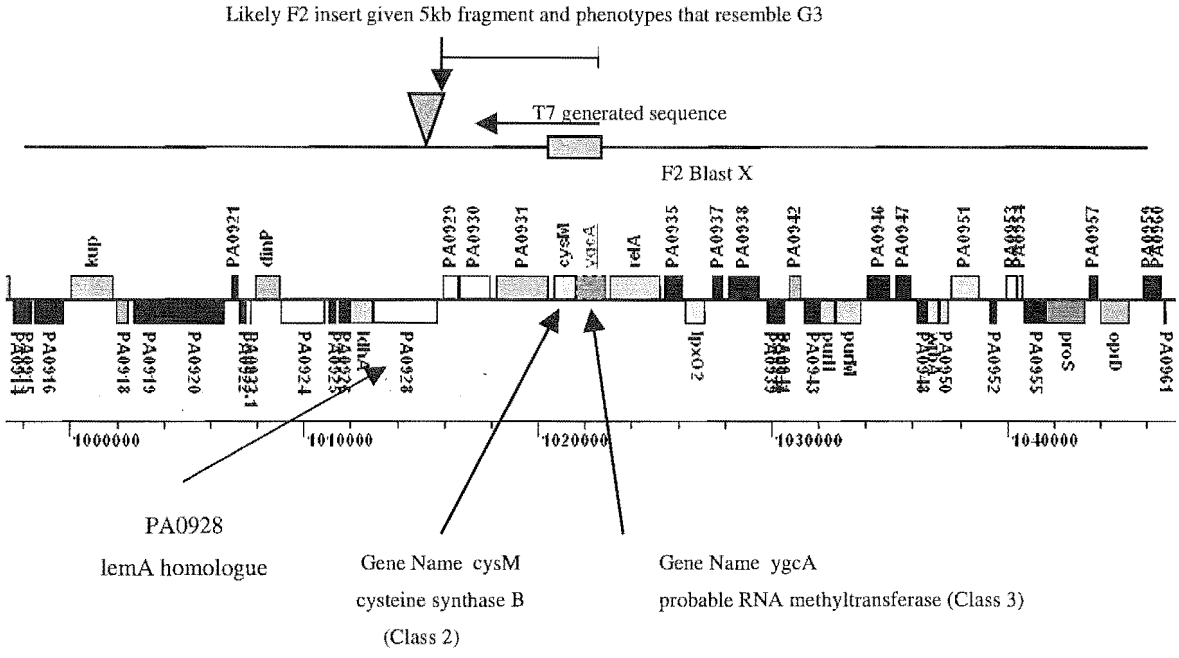


Figure 6-10 Spatial distribution of the mini-Tn*5*km*lacZ*2 insertion points of 103F2 within the suspected *gacS* analogue gene as depicted in *P. aeruginosa* genome annotation (http://www.pseudomonas.com).

6.3.3 Spatial relationship of Tn*5* insertions of p103E2 and p103D2

Although p103E2 and p103D2 shared the same phenotypic profile (Table 5-2), they were analysed independently as they were not suspected to be clonal based on isolation from different NZ103 restriction enzyme digested libraries. However, subsequent analysis revealed identical restriction maps, sequence identity and GenBank similarity (data not shown). The p103E2 and p103D2 mini-Tn*5*km*lacZ*2 insertion sites are likely to be very close, if not the same. Therefore, p103D2 alone is discussed here.

6.3.4 p103D2 (Bfm$^-$, Lip$^-$, Prot$^-$, M9$^+$, MJA^R, Mot$^+$, B2)

Characterisation of insert clone

A 10 kb *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was cloned from NZ103-D2 into pBluescript KS- to generate the plasmid pBSK-D2-2. Restriction analysis of pBSK-D2-2 indicated that ca. 5 kb and 100 bp genomic DNA was present on the *lacZ* and Km end of the transposon respectively (Figure 6-11).

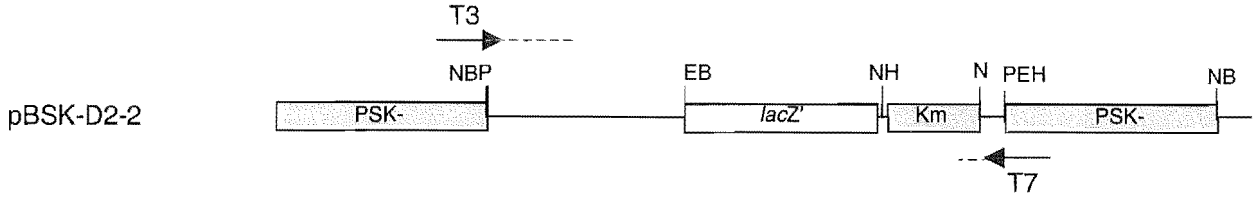


Figure 6-11 Schematic diagram of the pSK- clone derived from the NZ103 DNA flanking the mini-Tn*5*km*lacZ*2 insertion point of p103D2. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ*2

Nucleotide sequence for DNA flanking the *lacZ* end of mini-Tn*5*km*lacZ*2 within pBSKF2-1 was generated using the oligonucleotide primer T3 to obtain ca. 920 bp of sequence data (Genbank, AY224672). Nucleotide sequence with the T7 primer was not obtained.

Sequence comparisons to GenBank

The ca. 920 bp nucleotide sequence was found to have predicted translation products with similarity to 'transcriptional regulators belonging to the LysR family'. Of most relevance were the similarities to *P. aeruginosa* protein PA3895 (http://www.*Pseudomonas*.com, (Stover, *et al.*, 2000)) and *P. putida* KT2440 protein PP1262 (Nelson, *et al.*, 2002). This does not provide a great deal of resolution as to possible function as the LysR-type transcriptional regulators form a large family of bacterial gene activator proteins that control the expression of genes associated with a multitude of highly diverse cellular processes, ranging from amino acid biosynthesis, CO_2 fixation, ion transport, antibiotic resistance, initiation of nodulation, chromosomal replication and control of virulence. Therefore, it is likely that 103D2 mutation is within one of these LysR-type transcriptional regulators that has downstream effect on biofilm, protease and lipase production. Thus, the function of the inactivated gene in p103D2 is not clear from this sequence match.

6.3.5 p103F3 (Bfm+, Lip+, Prot+, M9-, MJA+, Mot+, B3)

Characterisation of NZ103 DNA insert clone

An ca. 10 kb *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was cloned from NZ103-F3 into pBluescript SK- to generate the plasmid pBK-F3-5. Restriction analysis indicated that approximately 2.0 kb and 3.0 kb of genomic information was present on the Km and *lacZ* end of mini-Tn*5*km*lacZ*2 respectively (Figure 6-12).

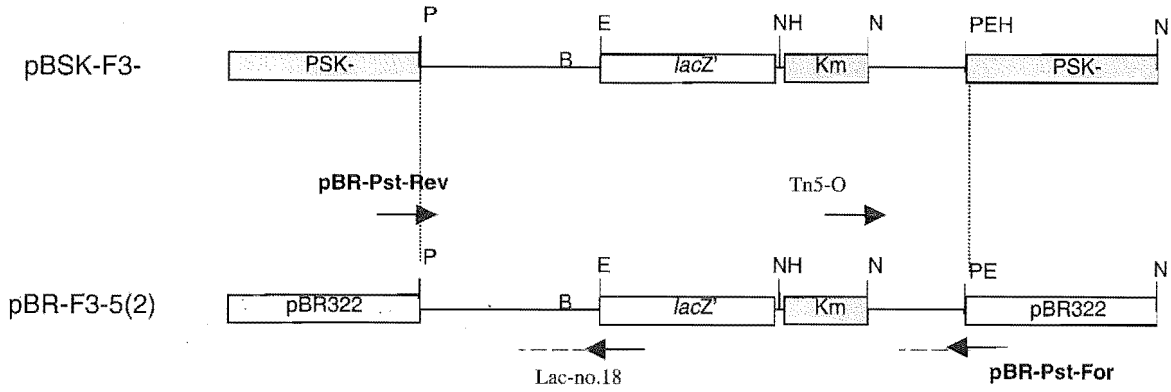


Figure 6-12 Diagram of the clone and subclone derived from NZ103 DNA flanking the mini-Tn*5*km*lacZ*2 insertion site of p103F3. Dashed lines indicate the fragment that was subcloned. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ*2

Because both flanking DNA fragments were >800bp, shotgun cloning was used to transfer the *Pst*I fragment containing mini-Tn*5*km*lacZ*2 into pBR322, resulting in the subclone pBR-F3-5(2). PCR amplification of DNA flanking the mini-Tn*5*km*lacZ*2 was achieved using oligonucleotides pBR-Pst-Rev/lac-no.18 and pBR-Pst-For/Tn*5*-O. Direct nucleotide sequencing of the PCR products was only successful with lac-no.18 from the pBR-Pst-Rev/lac-no.18 amplicon (ca. 800 bp, Genbank, AY224665), and Tn*5*-O from the pBR-Pst-For/Tn*5*-O amplicon (ca. 870 bp).

Sequence comparisons to GenBank

Nucleotide sequence comparison of pBR-Pst-Rev/lac-no.18 to GenBank indicated that pBR-F3-5(2) has similarity at the insertion point to the predicted translation product of a probable 2-hydroxyacid dehydrogenase (PA1296) from *P. aeruginosa* (http://www.*Pseudomonas*.com, (Stover, *et al.*, 2000)) and PP4589 of *P. putida* KT2440 (Nelson, *et al.*, 2002). Sequence obtained from the pBR-Pst-For/Tn*5*-O amplicon was obtained from pBR-Pst-For oligonucleotide primer and therefore did not sequence the insertion site. Nonetheless, this sequence gave similarity to predicted translation products for ribonuclease D gene *rnd* (e.g. *P. aeruginosa* PAO1 (gene PA1294)) which is suggested to

have involvement in transcription, RNA processing and RNA degradation. These two genes identified in p103F3 appear to be in the same gene order as in *P. aeruginosa* PAO1 (Figure 6-13).

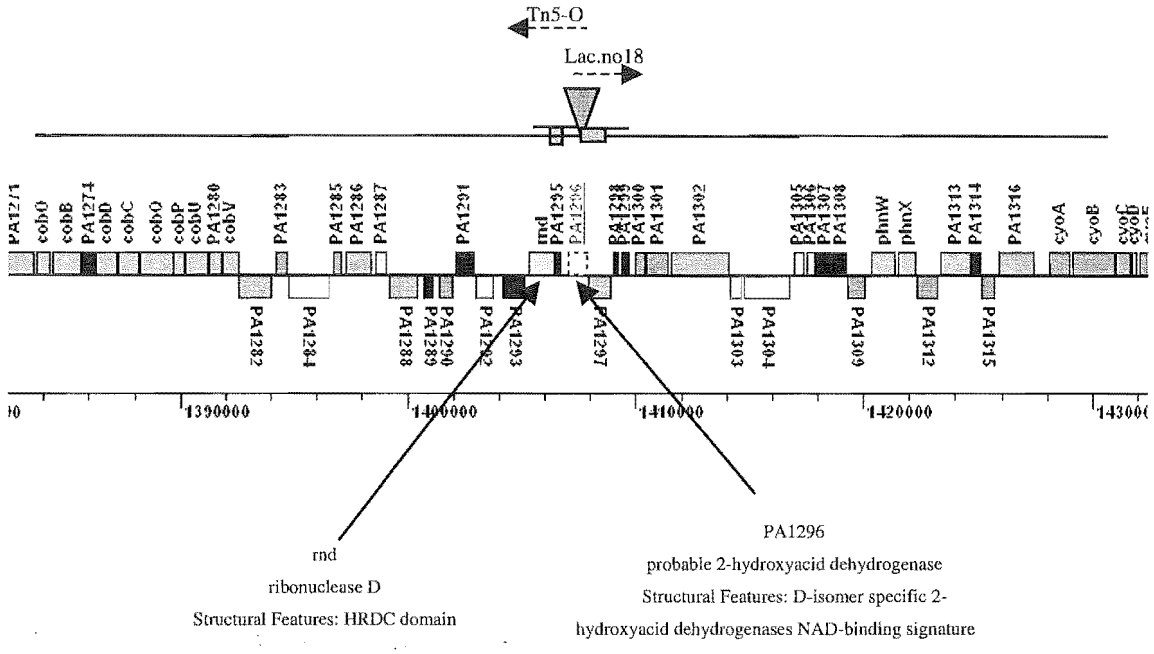


Figure 6-13 Spatial genetic arrangement of *P. aeruginosa* PAO1 *rnd* (PA1294) and PA1296 genes also suggest that the genetic arrangement in NZ103 is similar based on sequence analysis of p103F3 similarity of rnd and PA1296.

Comments

Nucleotide sequence comparisons suggest that in 103F3 the mini-Tn5km*lac*Z2 has inserted at 5' end of putative coding region of a 2-hydroxyacid dehydrogenase. As this gene is involved in catalyzing NADPH-dependent reductase activity for energy metabolism, it is most likely that this mutation has disrupted house keeping cellular metabolism. This is consistent with the phenotype in which it is unable to grow on M9 agar, but can within the nutrient rich LB broth containing digested protein and amino acid sources. Furthermore, the observation of blotch reduction suggests that 103F3 is unable to efficiently grow and reach the required cell-density threshold given only the nutrients that induce blotch, however growth on MJA where the colony was indistinguishable from the wildtype disputes this[1].

[1] The nutrient composition provided by MJA is suspected to be as efficient as those provided by LB as discussed in Chapter 4.9.1.

6.3.6 p103A5 (Bfm$^-$, Lip$^+$, Prot$^-$, M9$^+$, MJA$^+$, Mot$^+$, B2)

Characterisation of NZ103 DNA insert clone

An ca. 10 kb *Pst*I mini-Tn*5*km*lacZ2* containing fragment was cloned from NZ103-A5 into pBluescript KS- to generate the plasmid pBSK-A5-6. Restriction analysis of pBSK-A5-6 indicated that 3.3 kb and 1.8 kb of genomic DNA was present on the *lacZ* and the Km end of mini-Tn*5*km*lacZ2* respectively. The 3.3 kb fragment from pBSK-A5-6 was subcloned with BamHI to create pBSK-A5-3.3 (Figure 6-14).

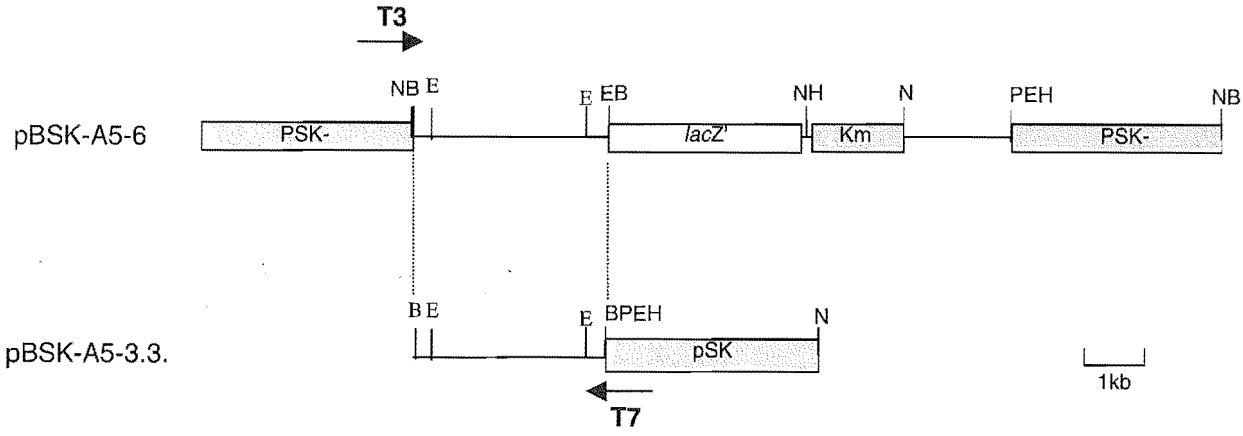


Figure 6-14 Schematic diagram of clone and subclone derived from the NZ103 DNA flanking the mini-Tn*5*km*lacZ2* insertion site of p103A5. Dashed lines indicate the fragment that was subcloned. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ2*

Nucleotide sequence from the transposon insertion point was obtained for the 3.3 kb subclone in pSK- directly using oligonucleotide sequencing primers T3 and T7 (Figure 6-14). Approximately 780 bp were recovered from each sequencing reaction (Genbank, AY224666).

Sequence comparisons to GenBank

Sequence obtained from the T3 primer gave predicted translation products with highest similarity to the Ypar31 gene product from *Pseudomonas alcaligenes* ATCC 55044 (Vaisvila, *et al.*, 2001). However, given the size of the DNA fragment flanking the mini-Tn*5*km*lacZ2* on the *lacZ* side, this sequence homology is not likely to be insertion point given predicted length protein and position in alignment. The similarity is interesting nonetheless as Ypar31 has been identified as an ORF within the *P. alcaligenes* super-integron In55044. Integrons are of continued interest because of their involvement in transfer of genetic material amongst bacterial species.

Integrons and superintegrons and their role in pathogenicity

As horizontal transfer is extensive amongst bacterial genomes (Jain, *et al.*, 1999), the discovery of integrons and antibiotic-resistance cassettes illustrates that bacteria have a natural genetic engineering tool to acquire potentially useful genes (Stokes and Hall, 1989). Integrons are genetic structures likely to facilitate rapid exchange of genes (Hall and Collis, 1995) and consist of arrays of promoterless gene cassettes separated by related DNA elements (59-base elements) that carry sites for integrases of the Int family (Nunes-Duby, *et al.*, 1998). Each integron has, at its 5' end, a gene for the relevant integrase, together with a promoter located within the integrase gene and oriented towards the gene cassettes (Levesque, *et al.*, 1994). Most of the known cassettes specify antibiotic resistance and are compact, most integrons have between one and three cassettes (Reechia and Hall, 1997).

Recently, a new type of integron, a super-integron was described as a chromosomal array of a large number of gene cassettes, mobilisable by a site-specific integrase obtained from an integron (Mazel, *et al.*, 1998). Despite the similarities, the super integrons are distinguished from conventional integrons in several respects including size, placement of promoters, replicon location and the nature of the genes found within the cassettes. Moreover, the functions encoded by the super-integrons are apparently diverse and some are possibly related to pathogenesis (Ogawa and Takeda, 1993, Mazel, *et al.*, 1998). Other proposed functions include transport of small molecules, restriction-modification, excreted lipase activity and plasmid specific roles (Barker, *et al.*, 1994, Barker and Manning, 1997, Rowe-Magnus, *et al.*, 2001). It has been proposed that *V. cholerae* superintegrons may function to cluster genes related to pathogenicity and to entrap genes specifying other biochemical functions that may respond to a variety of environmental signals (Rowe-Magnus, *et al.*, 2001). Pathogenicity-related genes appear to function to exploit a highly specific niche. A gene-capture system such as a super-integron appears well suited for acquisition of such niche-specific functions (Vaisvila, *et al.*, 2001). One could surmise that NZ103 is a BCO in an environment where horizontal transfer is likely to occur (see discussion in Chapter 8.5). Integrons and superintegrons may play a similar role in acquisition of gene(s) involved in virulence and formation of blotch. However, based on low translated protein similarity (36%) further analysis of 103A5 is required to substantiate the existence of integrons in NZ103.

6.3.7 p103F5 (Bfm$^+$, Lip$^+$, Prot$^+$, M9$^+$, MJA-, Mot$^+$, B3)

Characterisation of NZ103 genomic insert clone

As previously discussed in Chapter 5.9, special interest was placed in 103F5 because this mutant exhibited only one deficient phenotype[1], its inability to grow on MJA. This suggested that mini-

[1] Of the defined phenotypes tested in this study.

Tn*5*km*lacZ*2 insertion was within a gene that was directly or indirectly involved with the ability of NZ103 to grow on nutrients provided by *A. bisporus* mushroom tissue.

An ca. 8.7 kb *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was cloned from NZ103-F5 into pBluescript KS- to generate the plasmid pBSK-F5-2. Restriction analysis indicated that pBSK-F5-2 contained ca. 1.8 kb and 2.8 kb of genomic DNA on the *lacZ* and Km end of mini-Tn*5*km*lacZ*2 respectively (Figure 6-15).

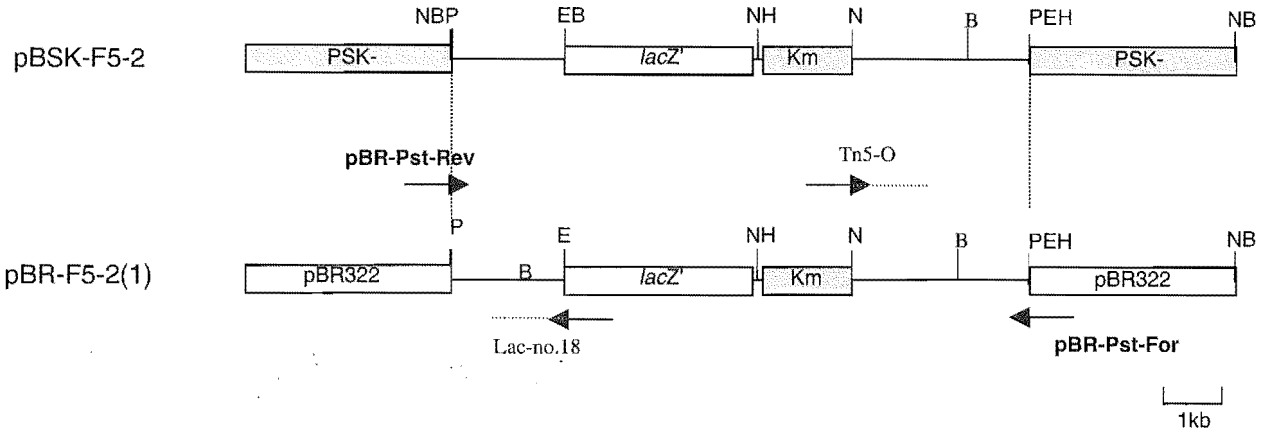


Figure 6-15a Schematic diagram of the *Pst*I clone of pBSK-F5-2. Dashed lines indicate the fragment that was subcloned into pBR322. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ*2

The *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was shotgun cloned into pBR322, resulting in the subclone pBR-F5-2(1). A PCR amplicon of NZ103 DNA at the *lacZ* end of mini-Tn*5*km*lacZ*2 was generated from pBR-F5-2(1) using oligonucleotides pBR-Pst-Rev/lac-no.18 (Figure 6-15a). Nucleotide sequence of the transposon insertion site was obtained by direct sequencing of the PCR amplicon using the oligonucleotide primer lac-no.18 to generate ca. 1 kb of sequence (GenBank, AY224667).

Sequence comparisons to GenBank

BlastX indicated pBR-F3-5(2) to have high similarity at the level of predicted translation products for the major outer membrane protein OprF of numerous *Pseudomonas* species including *P. syringae* (Ullstrom, *et al.*, 1991), *P. putida* KT2440 (Nelson, *et al.*, 2002), *P. viridiflava*, *P. cichorii* (Vermeiren, *et al.*, 1999) and *P. fluorescens* (De Mot, *et al.*, 1992).

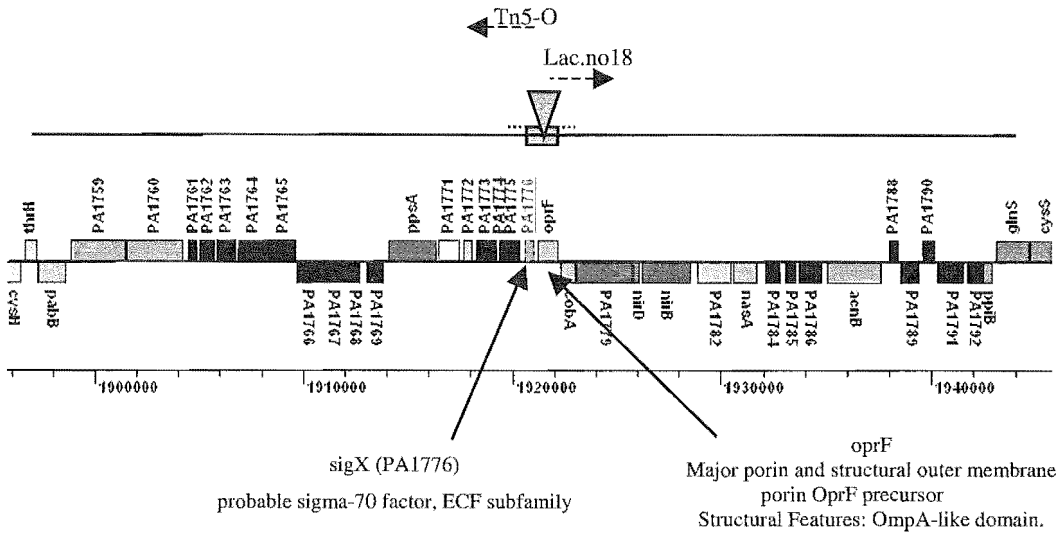


Figure 6-15b Insertion site of NZ103-F5 relative to *P. aeruginosa* PAO1 genome.

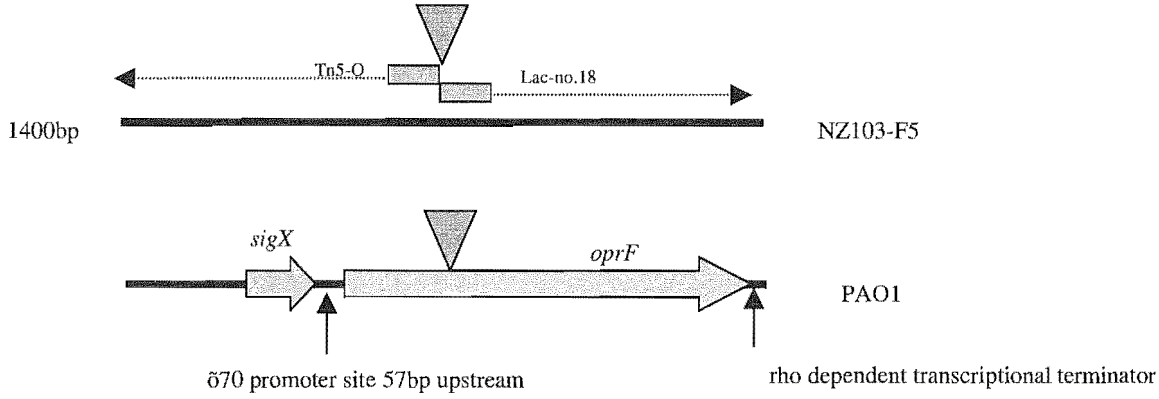


Figure 6-15c Predicted insertion site relative to the conserved oprF in numerous pseudomonads including: *P.. fluorescens; P.. viridiflava; P.. syringae; P.. putida; P.. cichorii.* Similar identification of sigX was observed in *P. syringae* AF115335.1 novel putative ECF (extracytoplasmic function) sigma factor gene, sigX

The outer membrane proteins, OmpA and OprF

Gram-negative pseudomonads are composed of two compartments, periplasm and cytoplasm, delineated by the cell wall outer membrane and the inner cytoplasmic membrane. The outer membrane of Gram-negative bacteria consist of two classes of macromolecules, lipopolysaccharides (LPS) and proteins. The cytoplasmic membrane is not rigid enough to support an excessive osmotic pressure gradient and this function is supplied by the cell wall outer membrane complex. The inner

membrane contains active transport systems and receptors that move material in and out of the cytoplasm and detect environmental signals (Cronan, *et al.*, 1987). Permeability of the outer membrane is largely determined by channels formed by porins such as *E. coli* OmpF and OmpC that facilitate movement of small hydrophilic solutes across the outer membrane (Hancock, 1987).

OprF, the major porin of *P. aeruginosa* is conserved among all serotypes (Mutharia, *et al.*, 1982) and has been extensively studied due to its proposed utility as a vaccine component, its role in antimicrobial drug resistance and its porin function (Benz and Hancock, 1981, Woodruff and Hancock, 1988, Hancock, *et al.*, 1990, Knapp, *et al.*, 1999). *P. aeruginosa* outer membrane protein OprF is a homolog of *E. coli* OmpA (Duchene, *et al.*, 1988, Woodruff and Hancock, 1989). OmpA is one of the major outer membrane proteins of *E. coli* but has very little pore-forming activity (unlike other major porins such as OmpF and OmpC (Hancock, 1987)). In contrast, *P. aeruginosa* OprF has been described as a bi-functional protein that has porin activity, forming small water-filled channels and a limited number of large membrane channels (Woodruff and Hancock, 1988).

The heterogeneity of major outer membrane proteins within a strain collection representing all biovars of *P. fluorescens* and *P. putida* was determined and an OprF-like protein was conserved among all strains using antibodies raised against OprF and western blot analysis (Kragelund, *et al.*, 1996). Other studies have also shown cell wall proteins that demonstrate relatedness to OprF and OmpA in other bacteria, including *Neisseria* sp. (Gotschlich, *et al.*, 1987), *Haemophilus influenzae* (van Alphen, *et al.*, 1983). Therefore, the high similarity shown between NZ103 OprF to other bacterial genera and pseudomonads was not surprising and suggests that NZ103 OprF will be highly conserved in structure and function.

OprF involvement in cell shape and osmolarity tolerance

OprF has been shown to be required for cell growth in low-osmolarity medium and maintenance of cell shape where OprF-deficient mutants have rounded morphology and grow well only in high-osmolarity media (Gotoh, *et al.*, 1989, Woodruff and Hancock, 1989). To determine whether the deleted gene(s) had similar properties as OprF, 103F5 was analysed by TEM to determine comparison of cell shape to that of NZ103 wildtype. It was observed that 103F5 cell shape was indeed exhibiting rounded morphology in comparison to NZ103 wildtype cell shape (Figure 6-16).

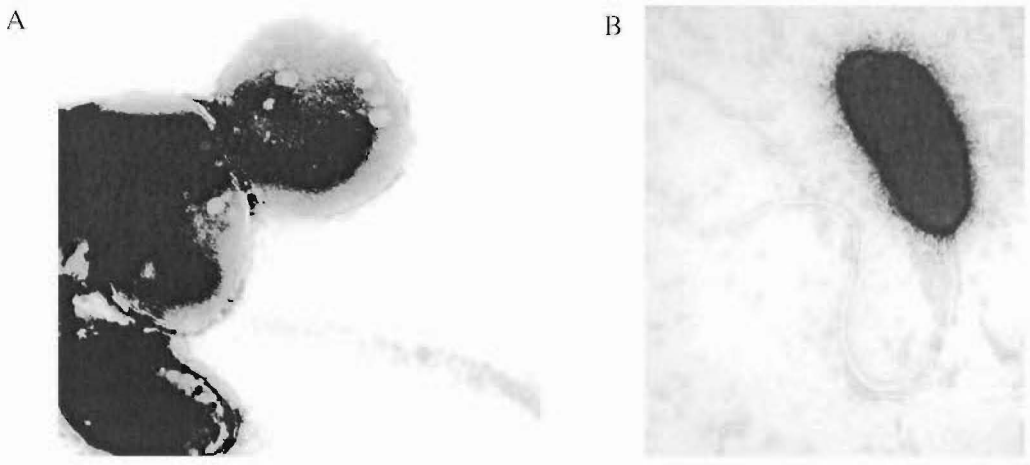


Figure 6-16 TEM analysis showing (A) the distinct rounded morphology of *Pseudomonas* 103F5 in comparison to (B) NZ103 wildtype cell.

Role of OprF in root adhesion

An OprF-like protein has recently been suggested to be involved in adhesion between a *P. fluorescens* strain and plant roots (De Mot and Vanderleyden, 1991, De Mot, *et al.*, 1992, De Mot, *et al.*, 1994). This possible role of OprF in adhesion combined with OprF involvement in tolerance in low osmotic environments suggest that the NZ103 *oprF* homologue identified in this study may not necessarily be involved in the blotch discolouration process, but likely involved in the ability of NZ103 to colonise and multiply on the *A. bisporus* tissue surface.

6.3.8 p103A14 (Bfm$^+$, Lip$^-$, Prot$^-$, M9$^+$, MJA$^+$, Mot$^+$, B3)

Characterisation of NZ103 insert clone

An ca. 6.5 kb *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was cloned from NZ103-A14 into pBluescript KS- to generate the plasmid pBSK-A14-5. pBSK-A14-5 was subsequently shown to contain ca. 1.3 kb and 400 bp of genomic DNA at the *lacZ* and Km end of mini-Tn*5*km*lacZ*2 respectively (Figure 6-17a).

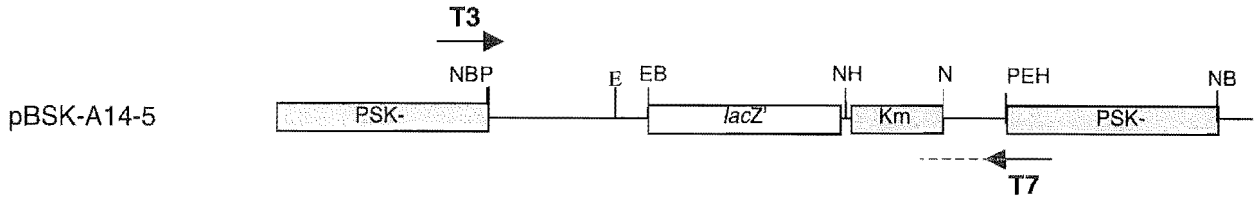


Figure 6-17a Schematic diagram of the *Pst*I clone derived from NZ103 DNA flanking the mini-Tn*5*km*lacZ*2 insertion site of p103A14. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lacZ*2

The *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was sequenced using the T7 oligonucleotide primer and provided nucleotide sequence of ca. 500 bp that encompassed the mini-Tn*5*km*lacZ*2 junction site (Genbank, AY224668).

Sequence comparisons to GenBank

Nucleotide sequence analysis indicated pBR-A14-5(4) to have similarity at the level of predicted translation products to a number of diverse proteins designated as having varying functions. Generally these functions were involvement in translocation of substances from within the cell to the outer surroundings. Publications containing genes with similarity to 103A14 include (Liao and McCallus, 1998, Idei, *et al.*, 1999, Kawai, *et al.*, 1999, Chabeaud, *et al.*, 2001).

Comments

Although the percentage identities were not high, two homologies were of particular interest. One was with *P. aeruginosa* PAO1 metalloprotease secretion protein *aprE* (PA1247, http://www.*Pseudomonas*.com) that resides in a cluster of genes (*aprD*, *aprE*, *aprF*, *aprA*, and *aprI*) that are involved in export of secreted cellular factors including proteins, toxins, enzymes, and alginate. This phenotype is consistent with the inability of 103A14 to produce proteases or lipases and it is likely that the mini-Tn*5*km*lacZ*2 insertion has disrupted cellular mechanisms for the excretion of these products. However, as alginate has been identified in biofilm formation (Costerton, *et al.*, 1987, De Vault, *et al.*, 1989, Davey and O'Toole, 2000), it is interesting that 103A14 still has a positive biofilm phenotype and suggests that the gene(s) responsible for alginate production have not been disrupted by mini-Tn*5*km*lacZ*2 insertion in 103A14 or, that alginate may simply not be involved in NZ103 biofilm formation.

The second translated protein homology of interest was the *eprE* gene from *P. tolaasii* because of the title of the unpublished manuscript "Characterisation of a gene cluster required for synthesis and secretion of extracellular metalloprotease by *Pseudomonas tolaasii*". Furthermore, this submission was from a prominent researcher of *P. tolaasii* brown blotch, namely Keith Johnstone of Cambridge

University. I was unable to find out any more information on the *eprE* gene, but can only surmise that it has functional similarity to the *aprE* metalloprotease secretion protein in *P. aeruginosa* PAO1. As was discussed earlier[1], metalloprotease production has been identified in *P. tolaasii* and suggested to be involved in brown blotch formation process (Baral, *et al.*, 1995).

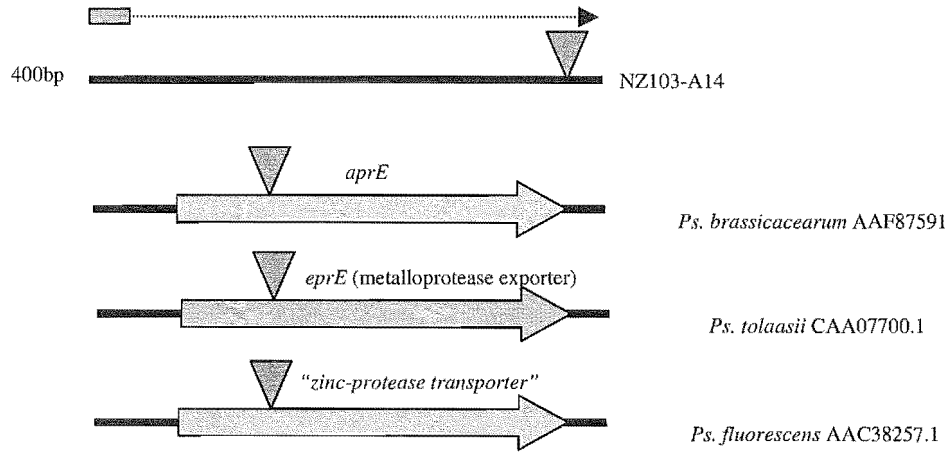
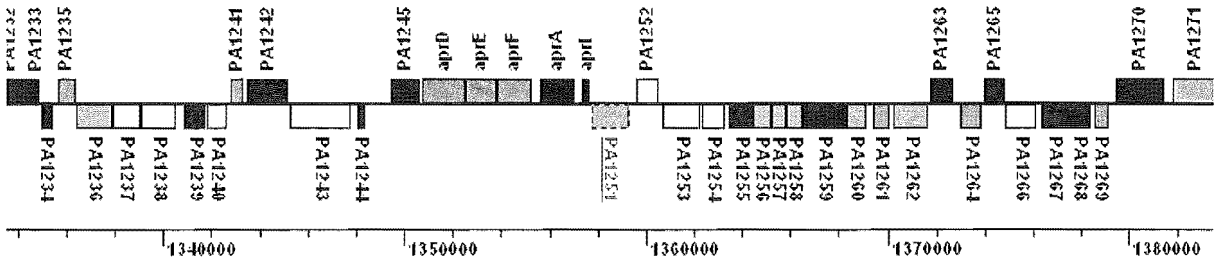


Figure 6-17b NZ103-A14 Tn*5* insertion site of BlastX identified similar genes.

Furthermore, when looking at the genetic order of the *aprE* cluster in *P. aeruginosa* PAO1 (http://www.*Pseudomonas*.com), it is clear that surrounding genes are heavily involved in cellular production and export and/or regulation of these functions. For example, genes in close proximity to the *apr* cluster (Figure 6-18) have homology to: two-component regulatory proteins (PA1243); a membrane bound protein (PA1245); and an *E. coli* methyl-accepting chemotaxis protein (PA1251).



[1] The metalloprotease produced by *P. tolaasii* is discussed in Chapter 4.4.3.

Figure 6-18 The genetic order of regulatory proteins in *P. aeruginosa* PAO1. The cluster of *aprD*, *aprE*, *aprF*, *aprA*, and *aprI* are described as having involvement in export of secreted cellular factors including proteins, toxins, metalloprotease, and alginate. PA1243 is a suggested two-component regulatory protein, PA1245 a membrane bound protein and PA1251 has homology to an *E. coli* methyl-accepting chemotaxis protein.

The mini-Tn*5*km*lac*Z2 insertion into an *aprE*-like gene in NZ103 may have caused polar mutational effects on any number of these regulatory proteins. Nonetheless, the complexity of cellular regulation of such functions as bacterial/fungal interaction and blotch formation is further shown by this example.

6.3.9 p103D13 (Bfm$^+$, Lip$^-$, Prot$^+$, M9$^+$, MJA$^+$, Mot$^+$, B4)

Characterisation of NZ103 DNA insert clone

An ca 8.7 kb *Pst*I fragment containing mini-Tn*5*km*lac*Z2 was cloned from NZ103-D13 into pSK- to generate the plasmid pBSK-D13. pBSK-D13 was subsequently shown to contain ca. 3.5 kb of genomic information and ca. 300 bp on the Km and *lac*Z end of mini-Tn*5*km*lac*Z2 respectively (Figure 6-19).

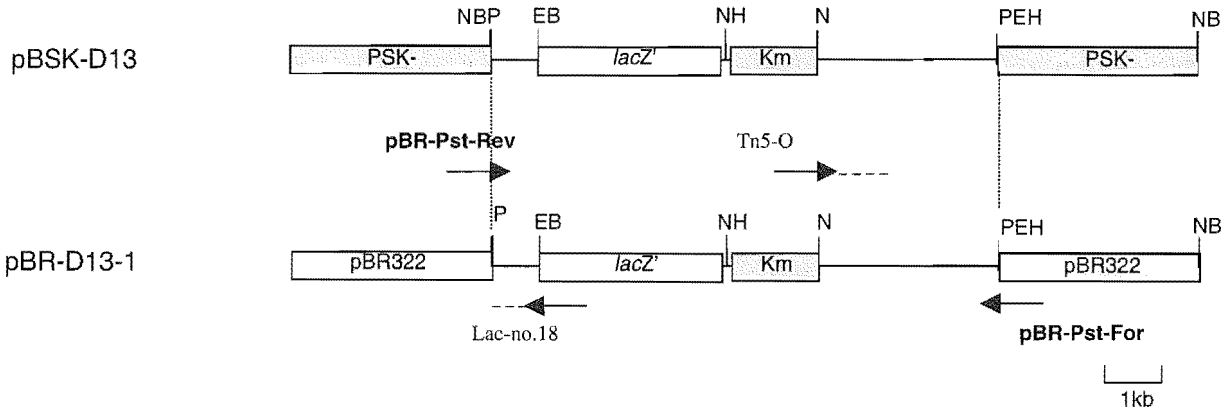


Figure 6-19 Schematic diagram of the *Pst*I clone derived from the NZ103 DNA flanking the mini-Tn*5*km*lac*Z2 insertion site of p103D13 and subsequent subclone. Dashed lines indicate the fragment that was subcloned. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

Sequencing of DNA flanking mini-Tn*5*km*lac*Z2

The *Pst*I fragment containing mini-Tn*5*km*lac*Z2 was excised and recloned into pBR322, resulting in the subclone pBR-D13-1. PCR amplification of the mini-Tn*5*km*lac*Z2 insertion site either end of mini-Tn*5*km*lac*Z2 was generated using oligonucleotide primers pBR-Pst-Rev/lac no.18 and pBR-Pst-For/Tn*5*-O. Nucleotide sequence was obtained for each amplicon using lac-no.18 (ca. 360 bp) and Tn*5*-O (ca. 860 bp, Genbank, AY224669).

Sequence comparisons to GenBank

Sequence analysis of the 860 bp from Tn*5*-O indicated pBR-D13-1 to have the highest similarity at the level of predicted translation products to probable glycosyl transferase proteins. These proteins were identified in genome sequencing projects (e.g. *P. fluorescens* 292[1], *P. putida* KT2440 protein PP4943 (Nelson, *et al.*, 2002) and *P. aeruginosa* PAO1 protein PA5004 (http://www.*Pseudomonas*.com)).

Furthermore, the probable glycosyl transferase PA5004 was amongst other *P. aeruginosa* PAO1 genes also heavily involved with membrane associated transferase functions, including the gene cluster *waaF*, *waaC*, *waaG*, and *waaP* involved in LPS cell wall biosynthesis. Furthermore, the probable glycosyl transferase similarity to 103D13 was also described as having involvement in LPS and capsule production. The mini-Tn*5*km*lacZ*2 may not have had a polar effect on these genes as biofilm formation has not been lost, but how this mutation affects lipase is left to speculation, perhaps the capsule and LPS are somehow involved in extracellular excretion of lipase.

6.3.10 p103A3 (Bfm$^-$, Lip$^-$, Prot$^-$, M9$^+$, MJA$^+$, Mot$^+$, B2)

Characterisation of clone

An ca. 7.5 kb *Pst*I fragment containing mini-Tn*5*km*lacZ*2 was cloned from NZ103-A3 into pBluescript KS- to generate the plasmid pBSK-A3-1. Restriction analysis of pBSK-A3-1 indicated that 1.2 kb and 2.3 kb of genomic DNA was present at the *lacZ* and Km end of mini-Tn*5*km*lacZ*2 respectively (Figure 6-20).

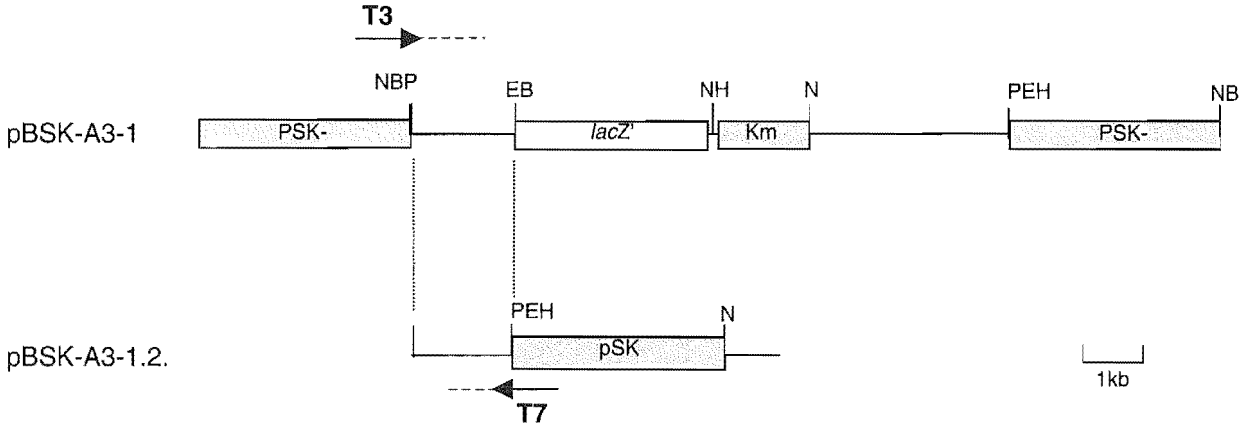


Figure 6-20 Schematic diagram of the pSK- clone and subclone derived from the mini-Tn*5*km*lacZ*2 insertion point of p103A3. Dashed lines indicate the fragment that was subcloned. Arrows indicate the origin and direction of sequencing. Abbreviations for restriction endonucleases; E, *Eco*RI; B, *Bam*HI; N, *Not*I; H, *Hind*III; P, *Pst*I.

[1] NCBI Microbial Genomes Annotation Project, National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

Sequencing of DNA flanking mini-Tn*5*km*lacZ2*

Sequence from the transposon insertion point was obtained by the generation of the subclone pBSK-A3-1.2, created by excising the 1.2 kb *Eco*RI/*Pst*I fragment originating from the *lac*Z end of the transposon and cloning back into pBluescript SK- (Figure 6-20). Nucleotide sequence spanning the 1.2 kb amplicon was obtained using T3 (ca. 768 bp) and T7 (ca. 970 bp) primers and the consensus sequence was submitted to GenBank (AY224664).

Sequence comparisons to GenBank

Sequence obtained from pBSK-A3-1.2 subclone gave predicted translation products with highest similarities to 'hypothetical proteins' with unassigned function, for example, from *P. fluorescens* (Accession # ZP_00085719) and *P. syringae* (ZP_00126523) (data not shown). However, sequence showed a lower homology to the lysine-, arginine-, ornithine (LAO) binding protein, a protein from *Salmonella typhimurium* (McClelland, *et al.*, 2001). LAO binding proteins are part of the extracellular solute-binding receptors of bacteria that serve as chemoreceptors, recognition constituents of transport systems, and initiators of signal transduction pathways (Tam and Saier, 1993). Furthermore, sequence similarity was also shown to *P. aeruginosa* PAO1 histidine transport system permease *hisQ* which lies within a cluster also containing *hisJ*, *hisM* and *hisP* which are considered membrane proteins that function in amino acid biosynthesis and metabolism and transport of small molecules. Matches to these genes may indicate that the reduction of blotch phenotype, blotch, lipase and protease production are due to an inability to export essential components across membranes and/or defects in the necessary signal transduction pathway to produce these phenotypes.

6.4 Summary of nucleotide sequencing of BCO^R clones

For each of the above cloned BCO^R mutants, information of the mini-Tn*5*km*lacZ*2 insertion site is summarised in Table 6-1. The amount of sequence obtained, GenBank accession numbers of reported matches, the regions of similarity, and the statistical significance of reported matches. Further to this, presented in Table 6-2 is a summary of the likely functional attributes associated with disrupted genes in each NZ103 mutant.

Table 6-1 Statistical information on the similarities reported for sequence obtained from selected biofilm minus and enhanced mutants.

Clone	Seq.(bp)	Match	Species	GenBank accession no.	Reg.(a.a)	Identity (%)	Positive (%)	E value
p103G3	1000	*rtpA*	*P. tolaasii*	BAA34717	263	71	79	2e-95
"	"	*gacS*	*P. chlororaphis*	AAK72489	263	73	79	2e-99
"	"	*ldhA*	*P. putida*	NP_743806	161	64	72	1e-95
P103F2	940	*cysM*	*P. aeruginosa*	A83530	85	85	94	6e-62
p103D2	920	PA3895	*P. aeruginosa*	NP_252584	34	70	82	4e-06
		PP1262	*P. putida*	AAN66886	39	76	89	3e-10
p103F3	800	PA1296	*Ps aeruginosa*	AAG04685	162	32	46	2e-17
		PP4589	*P. putida*	AAN70162	161	47	57	3e-32
	870	*rnd*	*P. aeruginosa*	AAG04683	67	74	89	2e-22
p103A5	780	*Ypar31*	*P. alcaligenes*	AAK73316	135	36	53	4e-14
p103F5	1100	*oprF*	*P. fluorescens*[1]	AAD22005	131	79	84	7e-75
p103A14	400	*aprE*	*P. brassicacearum*	AAF87591	96	62	70	7e-26
		aprE	*P. aeruginosa*	AAG04636	102	36	47	3e-06
		eprE	*P. tolaasii*	CAA07700	94	57	65	8e-22
p103D13	860	Pflu2880	*P. fluorescens*	ZP_00085620	260	93	94	1e-138
		PA5004	*P. aeruginosa*	AAG08389	258	29	48	7e-23
p103A3	1200	*LAO*	*Salmonella typhimurium*	AAL21256	74	52	64	2e-19
		hisQ	*P. aeruginosa*	AAG06312	138	55	71	3e-43

Description of terms

Seq. : Amount of sequence obtained

Match : The match to predicted translation products of referenced genes on the BlastX server

Region : The length of similarity that was reported in amino acids.

Identity : The percentage of predicted amino acid residues from the submitted sequence that give an identical match to those of the referenced sequence.

Positive : As with identity, but also includes in the calculation those predicted amino acid residues form the submitted sequence that although different to those of the referenced sequence are known to have similar chemical properties.

E value : A statistical measure of the likelihood that the reported match may have occurred by chance.

GenBank : The reference number for this sequence on the BlastX server accession

[1] Note that many other pseudomonad OprF homologs exhibited near-equal similarity to p103F5 sequence.

Table 6-2 Further summary of selected genotypically characterised NZ103s mutants, the accession number of deposited sequence from this study to GenBank, gene(s) with similarity to the gene disruption site, and that gene(s) known functionality in reported matches contained in GenBank.

Clone	Accession #	Gene homology	Functional description / comments
p103G3	AY224670	*rtpA*	Two component regulatory sensor kinase identified in *P. tolaasii* pathogenicity
"	"	*gacS*	Two-component regulatory proteins involved in regulation by autophosphorylation in response by an environmental signal(s).
P103F2	AY224671	*cysM*	Spatially located in *P. aeruginosa* PAO1 genome near *gacS* regulatory genes – given phenotype of 103F2, likely a polar mutation affecting *gacS* genes
p103D2/E2	AY224672	PA3895	Homology to LysR family of transcriptional regulatory proteins.
p103F3	AY224665	PA1296	Probable 2-hydroxyacid dehydrogenase. Insertion site likely to be involved in cellular metabolism.
p103A5	AY224666	*Ypar31*	An ORF homologue previously identified within *P. alcaligenes* super-integron In55044. Low match but interesting nonetheless.
p103F5	AY224667	*oprF*	Outer membrane protein F involved in osmolarity regulation, cell shape and root colonisation.
p103A14	AY224668	*aprE / eprE*	Metalloprotease secretion proteins. Spatially located in *P. aeruginosa* PAO1 amongst other genes involved in export of proteins, toxins, enzymes and alginate.
p103D13	AY224669	PA5004	Homology to glycosyl transferase.
p103A3	AY224664	*LAO*	Lysine-, arginine-, ornithine- binding extracellular proteins that may act as chemoreceptors, transport systems and initiators of signal transduction pathways

CONCLUSIONS

Transposon mutagenesis of the selected BCO, NZ103, generated 45 mutants deficient in one or a combination of PPDs (Table 5-1). These mutant isolates were further analysed to determine the effect that the mini-Tn*5*km*lacZ*2 insertion had on the ability of these strains to form blotch discolourations in the mushroom cube bioassay. Ten mutants with severe reduction in blotch (B4 or less) and that exhibited variable PPDs were identified (Table 5-2). These mutants were further characterised by nucleotide sequence analysis of the insertion sites to enable comparison to known nucleotide sequences and proteins identified in GenBank. This similarity yielded many interesting findings which have been placed in context with current understanding of bacterial pathogenicity and bacterial environmental physiology.

6.5 Overview of types of similarities found in the selected NZ103mutants

As summarised in Table 6-1, NZ103 genes interrupted by mini-Tn*5*km*lacZ*2 gave varying amounts of gene similarities when compared to submissions in GenBank. Some of these genes have been extensively studied in respect to their functional involvement within the bacterial cell whereas other gene descriptions are no more than 'hypothetical proteins' predicted from large-scale genome sequencing bioinformatic projects. Therefore, some genes have proved more interesting discussion points than others. Mini-Tn*5*km*lacZ*2 was assumed to have inserted within the genome of NZ103 based on similarities of insertion sequences to the genomic organization of *P. aeruginosa*. However, without Southern hybridisation data of genomic DNA and plasmid extracted DNA from NZ103 mutants, it cannot be excluded that insertion may have occurred within extragenomic fragments.

6.6 Mutants with similarity to two-component regulatory proteins

One of the themes to come out of this study was the likely role of two-component systems in regulation of processes required for blotch disease. Specifically, p103G3, p103F2, p103E2 and p103D2 showed translational and nucleotide similarity to *P. tolaasii rtpA* and *P. chlororaphis gacS*. p103A3 showed similarity to LAO, a suspected protein also involved in signal transduction pathways. Information relating to these individual genes has been discussed under the respective subheading, but it is interesting to observe that these abovementioned mutants exhibited deficiencies in more than one phenotype tested which is concurrent with previously described mutations of two-component regulatory systems (Heeb and Haas, 2001). It was striking that these mutants exhibited almost a total loss in blotch forming ability. This suggests that because growth rates were not affected by the mini-

Tn*5*km*lacZ*2 insertion[1], that the two-component regulatory proteins play very important roles in the cellular function of NZ103 in either colonisation and survival, and/or induction of blotch discolouration of *A. bisporus* tissue.

6.6.1 The importance of two-component regulatory systems in cellular functions

In some ways, the isolation of mutants in two-component regulatory pathways provides validation for the approach taken to determine molecular factors involved in blotch disease. The basis for this being that two-component regulatory proteins have been extensively implicated in such important pathways including chemotaxis, nitrogen and phosphate regulation, osmoregulation, secretion of extracellular proteins, flagella biosynthesis and virulence (Stock, *et al.*, 1989). Two-component regulatory systems modulate gene expression via a system that generally consists of a sensor-kinase and a response regulator, that use reversible protein phosphorylation to regulate the adjustment to environmental stimuli (Parkinson and Kofoid, 1992). Sensors are generally integral membrane proteins that respond to specific environmental signals by phosphorylating their cognate regulators, which are often transcriptional factors whose affinity for DNA is modulated by phosphorylation. However, it is not surprising that two-component regulatory proteins were identified in BCO given that homologs are found in both Gram-negative and Gram-positive bacteria, including the genera *Agrobacterium, Bacillus, Bordetella, Enterobacter, Escherichia, Myxococcus, Rhizobium, Staphylococcus, Salmonella* and *Pseudomonas*. It has been estimated that some bacteria harbor up to 40 different types of signal transduction systems responding to a variety of chemical and physical signals (Stock, *et al.*, 1995).

P. tolaasii PheN (another two-component regulatory protein) involvement in colony transition

As previously discussed in Chapter 2.4.2, the *pheN* locus was identified in *P. tolaasii* and shown that a functional copy of the *pheN* gene was required to maintain the smooth pathogenic phenotype, whereas the loss of the *pheN* gene (or its function) results in conversion to the rough phenotype (Grewal, *et al.*, 1995). The protein encoded from the 2727 bp ORF (Han, *et al.*, 1997) from the *pheN* locus shows similarity to members of a family of the two-component regulatory proteins (Figure 6-21). This similarity was determined through sequence comparisons that revealed the presence of both histidine protein kinase (HPK) and response regulator (RR) domains (Grewal, *et al.*, 1995).

[1] Growth rate determination was carried out in LB for mutants 103G3, 103 D2 and 103F2 (Figure 5-6).

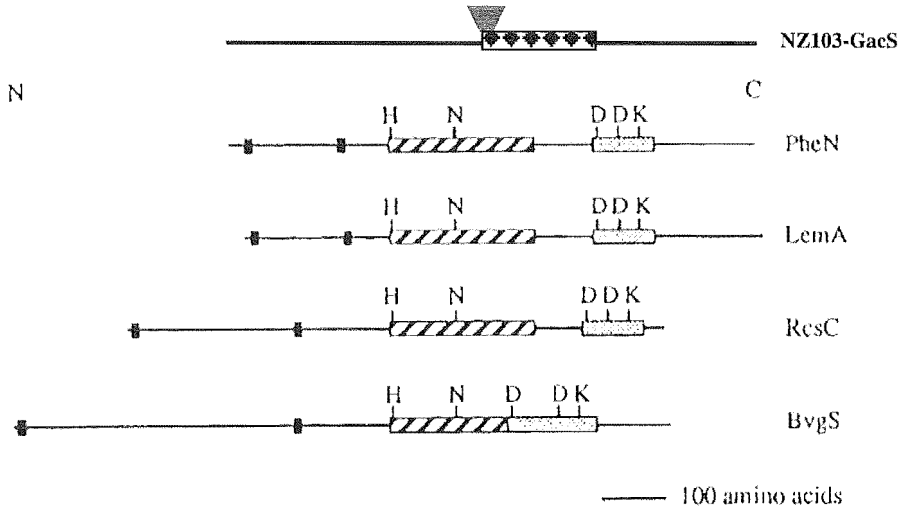


Figure 6-21 Schematic alignment (Grewal, *et al.*, 1995) of the predicted *P. tolaasii* PheN protein with *P. syringae* LemA, *E. coli* RcsC, and *B. pertussis* BvgS proteins. Small black squares, hydrophobic domains; hatched boxes, regions with homology to the conserved histidine protein kinase domain; stippled boxes, regions with homology to the conserved response regulator domain. Conserved amino acids are marked above the conserved histidine protein kinase and response regulator domains. NZ103-gacS is also included and the prediction insertion site relative to PheN is depicted by red triangle and box shows BlastX similarity from oligonucleotide primer Lac.no18.

The protein encoded by *pheN* contains two hydrophobic domains in the cytoplasmic membrane with its carboxy-terminal transmitter and regulator domains located in the cytoplasm. The amino-terminal extra-cytoplasmic domain of the *pheN* protein may sense the environment and transfer signals to its carboxy-terminus response regulator module via the transmitter domain. The response regulator domain then controls the expression of other genes either directly or via a second unknown component. This phenotypic switch may be regulated at three different levels. First, in the presence of stimulatory environmental signals, either *pheN* activates directly or indirectly the expression of the genes involved in tolaasin synthesis, protease production, and mucus production, but represses certain other genes controlling siderophore production, motility and chemotaxis. Second, the presence of autoregulatory compounds at a threshold concentration and poor nutrient conditions might completely eliminate expression of the *pheN* gene itself and result in the appearance of the highly unstable phenotypic rough form which appears around the margins of smooth colonies and reverts back to the smooth form when subcultured onto fresh medium. Third, the appearance of the metastable phenotypic rough form, which arises as sectors at the margins of smooth colonies, is a result of DNA rearrangements associated with the *pheN* locus.

As two-component regulatory proteins have been established to coordinately regulate the expression of multiple genes in response to environmental conditions, the phenotypic variation in *P. tolaasii* under the regulation of PheN could be explained as a detection of depletion of nutrients when cell density is high. Nutrient stress and production of bacterial secondary metabolites in turn may positively affect pheN expression, thus the smooth phenotype exhibiting pathogenicity prevails giving

a selective advantage. Although the presence of PheN was not investigated in NZ103, the similarity of 103G3, 103D2, 103E2 and 103F2 to *gacS* and *rtpA* suggests a regulatory system homologous to PheN in NZ103. A comparative study of NZ103 and *P. tolaasii* PheN would benefit understanding of this involvement in pathogenicity.

Two-component regulatory proteins involved with the extracellular production of proteins

Two-component regulatory proteins have been implicated in the regulation and production of extracellular proteins. For example, *B. subtilis* secretes a number of enzymes, including amylases and proteases during stationary phase and the expression of these secreted proteins has been shown to be globally regulated at the transcriptional level by at least four genes (reviewed in (Stock, *et al.*, 1989)). *P. aeruginosa* produces the exopolysaccharide alginate, resulting in mucoid colony forms involved in infection and biofilm formation regulated by *algR* and *algD* (De Vault, *et al.*, 1989). In *Staphylococcus aureus* proteins are expressed under the pleiotropic regulation of the gene (*agrA*) in stationary-phase cultures including serine protease, nuclease, lipase, fibrinolysin, α-, β-, and δ-hemolysin and enterotoxin B (Mallonee, *et al.*, 1982). This could also explain why p103G3, p103F2, p103E2 and p103D2 all exhibit deficient protease and lipase phenotypes (Table 5-2). It is likely that the RtpA/GacS two-component regulatory protein homolog in NZ103 is responsible for the regulation of many phenotypes that include the expression and/or extracellular translocation of extracellular proteins.

As many pseudomonads associated with mushroom production produce EPS (Fett, *et al.*, 1995) and it is thought that EPS may provide both protection from adverse environmental conditions (Singh, *et al.*, 1992, Osphir and Gutnick, 1994) and a means for attachment to fungal mycelia (Rainey, 1991), it is likely that EPS production is a requirement of biofilm formation. This suggestion is concurrent with observations in this study in which the wildtype NZ103 is produces a highly mucoidy colony phenotype and NZ103 biofilm mutants were noticeably less mucoidy. Furthermore, TEM observations showed that the outer cell wall staining, assumed to be the EPS was less in biofilm mutants and enhanced in 103B5++. With these observations, either EPS is involved in biofilm formation, or the mutations have affected genetic pathways that control multiple gene functions that includes another genetic factor responsible for biofilm reduction, and EPS is only an artifact of this mutation. To confirm this would require further investigation focusing in on EPS production.

Interaction of two-component regulatory systems and quorum-sensing

The involvement of quorum-sensing, and in particular *N*-acyl-homoserine lactones (AHLs), in biofilm formation have been reviewed in Chapter 4.3.3. It is increasingly evident that two-component regulatory systems and AHL-mediated regulatory systems rarely function independently. Instead, they are components of complex regulatory signal cascade mechanisms (Pierson III, *et al.*, 1998). A hierarchical cascade regulating *P. aeruginosa* PAO1 elastase production includes the LasR/LasI and RhlR/RhlI AHL-mediated response systems, as well as the alternate sigma factor RpoS (Latifi, *et al.*,

1996). The production of N-butyryl-homoserine lactone (BHL), the cognate signal of the RhlR/RhlI system, is reduced or delayed in *gacA* mutants of strain *P. aeruginosa* PAO1 (Reimmann, *et al.*, 1997). Furthermore, *P. aureofaciens* 30-84 showed that the GacS/GacA two-component regulatory system controls the production of AHL required for phenazine production (Chancey, *et al.*, 1999). Although quorum-sensing has not yet been shown to be involved in NZ103, it is tempting to speculate that it may be present based on the identification of 103B5++, the biofilm enhanced mutant (Chapter 4). Based on these observations and speculation, both biofilm production and the ability to induce blotch may be likely due to the combination of a GacS/GacA/RtpA homolog that also regulates quorum-sensing, such as AHL in *P. aeruginosa*. This would again prove a very interesting area for future research into the coordinated cellular signaling amongst environmental bacteria.

6.7 OprF involvement in survival of NZ103 on sporophore nutrients

The inability of 103F5 to grow on MJA suggested that mini-Tn*5*km*lacZ*2 had caused a mutation preventing the utilisation of available nutrients from the mushroom. The insertion site of mini-Tn*5*km*lacZ*2 in 103F5 had high translational similarity to the major outer membrane OprF from pseudomonads such as *P. aeruginosa* (Hancock, 1987), *P. syringae* (Ullstrom, *et al.*, 1991), *P. putida* (Nelson, *et al.*, 2002), *P. viridiflava*, *P. cichorii* (Vermeiren, *et al.*, 1999) and *P. fluorescens* (De Mot, *et al.*, 1992). *P. aeruginosa* OprF was found to play a role in cell shape and TEM studies of 103F5 also showed that cell shape was altered (Figure 6-16) and displayed rounded morphology as described in previous studies (Gotoh, *et al.*, 1989, Woodruff and Hancock, 1989). This would suggest that OprF in NZ103 has similar roles to *P. aeruginosa* OprF, providing porin activity, and forming small water-filled channels across the membrane (Woodruff and Hancock, 1988). Such channels are likely to be involved in ion-exchange across the membrane and although the composition of mushroom tissue has identified the presence of ions (Mau, *et al.*, 1993), it is unlikely that osmolarity of the mushroom environment is higher than within a bacterial cell. Therefore, porins such as OprF are likely to balance osmotic pressure by regulation of solutes in and out of the periplasmic space, and an absence of such proteins would mean an unregulated trans-membrane osmotic pressure in which water would move from the less ion rich environment (the mushroom / MJA) to the bacterial cell. This would explain the observation of OprF mutants showing rounded morphology in that water is entering the bacterial periplasmic space driven by osmolarity that the mutated 103F5 OprF is unable to regulate. Furthermore, the observation of OprF deficient bacteria requiring high osmolarity medium also supports this role, as an external medium with high ion content would contribute to balancing osmolarity across the bacterial cell wall. Growth of 103F5 was observed on both LB and M9 and this is likely due to both media having high salt content that would similarly prevent osmotic stress and cell rupture. However, TEM observations were of 103F5 cultured on LB agar, and it was evident that cellular swelling had occurred even given the relative high salt content of LB. The initial observation

of an absence of growth by 103F5 on MJA is suggestive that there are not enough ions present in the mushroom sporophore to prevent cellular rupture of the osmoregulation-compromised bacterial cells. Although OprF is likely a protein that is primarily involved in intracellular cell integrity maintenance and its mutation in 103F5 has caused a reduction in blotch simply because the bacteria is unable to grow on the osmotic environment provided by the mushroom tissue, it has further shown the importance of bacteria fitness in the processes of causing pathogenicity. Furthermore, this example stresses the limitation that *in vitro* bioassays have in totally representing a given environmental condition, if MJA had not been used, this mutation would not have been detected on either M9 or LBA.

In summary, given that: (i) OprF is required for cell growth in low-osmolarity medium and for maintenance of cell shape (Gotoh, *et al.*, 1989, Woodruff and Hancock, 1989); and (ii) OprF has been suggested to be involved in adhesion between a *P. fluorescens* strain and plant roots (De Mot and Vanderleyden, 1991, De Mot, *et al.*, 1992, De Mot, *et al.*, 1994); one could conclude that OprF seems to be important for NZ103 colonisation of *A. bisporus* and/or formation of blotch.

6.8 Homology with metalloprotease secretion proteins

NZ103-A14 mutant homology with metalloprotease secretion proteins in *P. aeruginosa* PAO1 was interesting given that the most prominent BCO, *P. tolaasii*, has also been found to produce an extracellular proteinase very similar to those secreted by other *Pseudomonas* species (Fairbairn and Law, 1986). The effect of this *P. tolaasii* metalloprotease in the infection of the mushroom sporophores is still unknown but has been speculated by other authors to contribute to brown blotch of *A. bisporus* (Baral, *et al.*, 1995). Although it is likely that the metalloprotease secretion proteins are only likely to be in NZ103 and other protease producing BCOs (Table 4-2), excretion of extracellular compounds is likely to be important in blotch. Given the spatial location of *aprE* in *P. aeruginosa* PAO1 amongst other genes involved in export of proteins, toxins, enzymes and alginate suggests that regulation and expression of *other* genes involved in production and export of such compounds contribute to blotch formation. Regulation and co-expression of these genes would also prove an interesting future area of investigation to gain further understanding into the contribution that extracellular factors contribute to blotch formation.

6.9 Final conclusion of transposon mutagenesis

The Tn*5*-disrupted genetic regions identified in this section of study provide a valuable insight into genetic components that are important in *A. bisporus* colonisation and/or blotch discolourations. Although no genes were fully characterised in this chapter, similarity to genes identified in previous studies will provide a foundation that future research can build upon to further characterise and understand bacterial/fungal interactions and/or the development of blotch disease.

Chapter 7

OVERALL CONCLUSIONS AND DEVELOPMENT OF BACTERIAL BLOTCH MODELS

Specific areas of research undertaken and the findings obtained have been addressed in detail within the respective chapters presented in this study. A holistic overview of the results obtained in this thesis has established that bacterial blotch discolourations are a result of a multitude of complex interacting processes; highlighted by; (i) many varying bacterial blotch discolourations could be caused by many different pseudomonads; (ii) no common pathogenicity determinant was found amongst BCOs that showed conclusive involvement in blotch discolourations; and (iii) transposon mutagenesis identified complex mechanisms involved in genetic regulation of multiple phenotypes. These three levels of complexity (bacterial, phenotypic, and genetic) prompted the development of models that help rationalize the contextual placement of knowledge gained within this thesis, with that of which is already in the literature. The following sections contain models that are considered to outline the important factors involved in the blotch discolouration processes of *A. bisporus*.

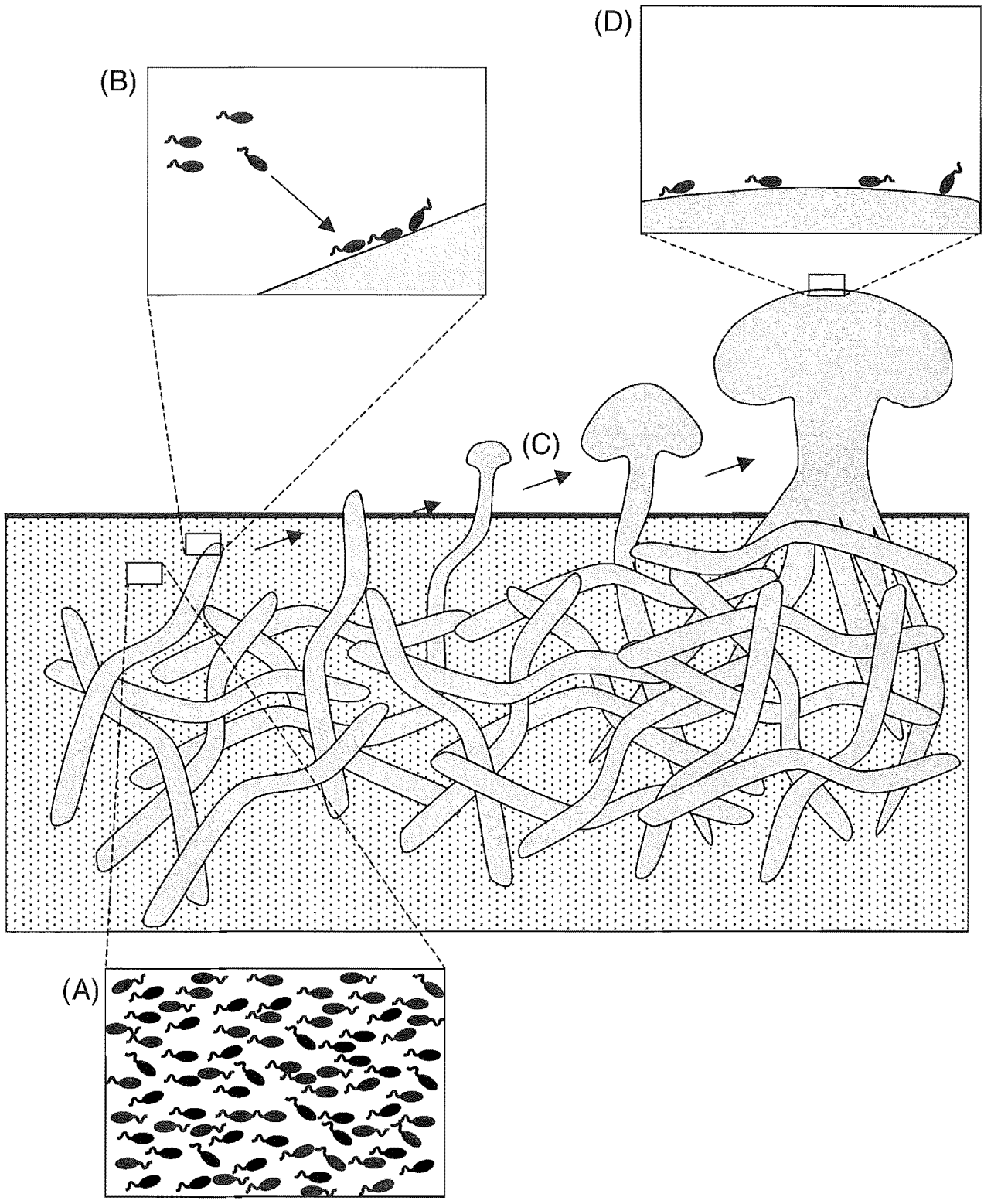
Because this chapter is essentially a review of all information presented in this thesis, individual literature references are not cited, but rather, reference is made of the Chapter/Section in which the observation has been discussed previously.

BACTERIAL BLOTCH MODELS

7.1 Model 1 Bacterial colonisation of *A. bisporus*

There is an abundant natural microflora associated with the cultivation of *A. bisporus*[1] and microbial interactions have a range of effects on *A. bisporus*[2]. Amongst this microflora, pseudomonads are present in vast quantities throughout the compost and casing[3] (Model 1-A). Growing *A. bisporus* mycelia in the casing and compost produce exudates that cause chemotactic response by the motile pseudomonads[4] (Model 1-B). They will subsequently adhere to the mycelial tissue and will remain adhered as the mycelia develop into sporophores (Model 1-C), therefore, bacteria will be present on the mature mushroom cap (Model 1-D), but at this stage, bacterial numbers are low and they elicit no disease symptoms.

[1] Chapter 1.1.3.
[2] Chapter 1.3 and Chapter 1.4
[3] Chapter 1.4.5 and Chapter 2.3
[4] Chapter 1.5.7

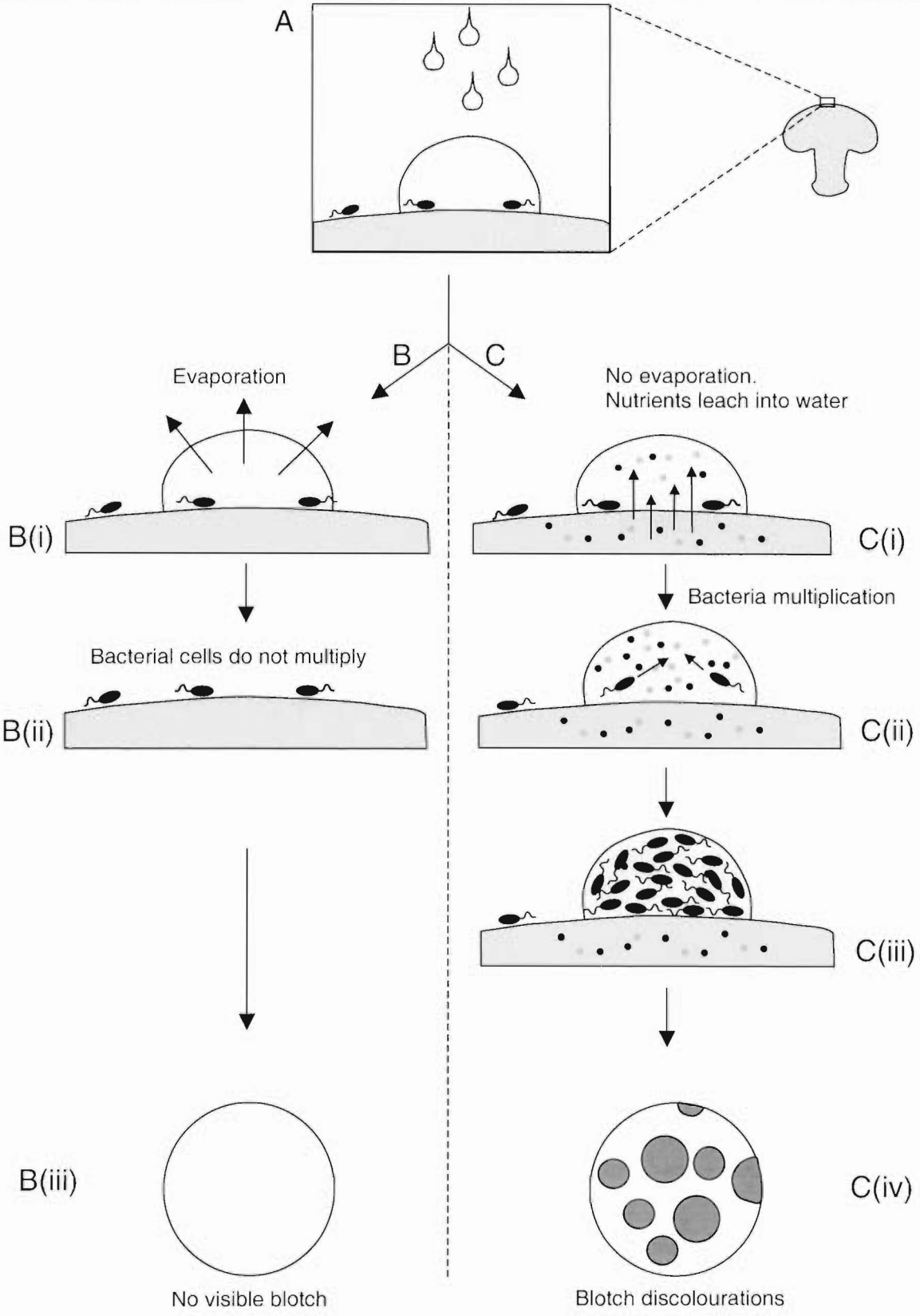


Model 1 (A) Pseudomonads are present in high cell densities within compost and casing; (B) Bacterial colonisation occurs on growing hyphae in the compost and casing material via chemotaxis response, flagellar mediated motility, and adherence via pili and extracellular polysaccharides; (C) Bacterial cells remain attached to the mycelial surfaces during the formation of sporophores; and (D) these bacterial cells are in low numbers and generally do not elicit disease.

7.2 Model 2 Physiological causes of blotch formation

During mushroom cultivation, processes such as altering humidity, over watering, and natural changes in environmental temperatures, all promote condensation and water droplet formation on the mushroom cap[1] (Model 2-A). The presence of this water droplet is a critical factor that ultimately determines blotch severity. Correct farm management involves manipulation of environmental conditions to promote evaporation (Model 2-B) that removes the water (Model 2-Bi) and the bacteria present on the mushroom cap are unable to multiply exponentially (Model 2-Bii) and thus, no blotch is observed (Model 2-Biii). However, if condensation is not removed, water droplets will remain on the mushroom cap (Model 2-C) and the nutritionally rich components from the mushroom tissue will leach into the water droplet via osmotic diffusion (Model 2-Ci). The water droplet fast becomes a 'nutritional soup' and bacterial cells within this water droplet are provided with the nutritional requirements for exponential growth (Model 2-Cii). This rapid growth generates a bacterial cell-density threshold (Model 2-Ciii) and it is this threshold that has been widely shown to be essential for blotch formation[2] (Model 2-Civ). Cell-density was also shown to be necessary for blotch formation by the BCOs in this study[3]. However, because results showed not all pseudomonads isolated could cause blotch discolourations[4], this model is therefore only true for pseudomonads that have blotch-inducing factors (BIFs).

[1] Chapter 1.5.5
[2] Chapter 1.5.6
[3] Chapter 2.9
[4] Table 2-2



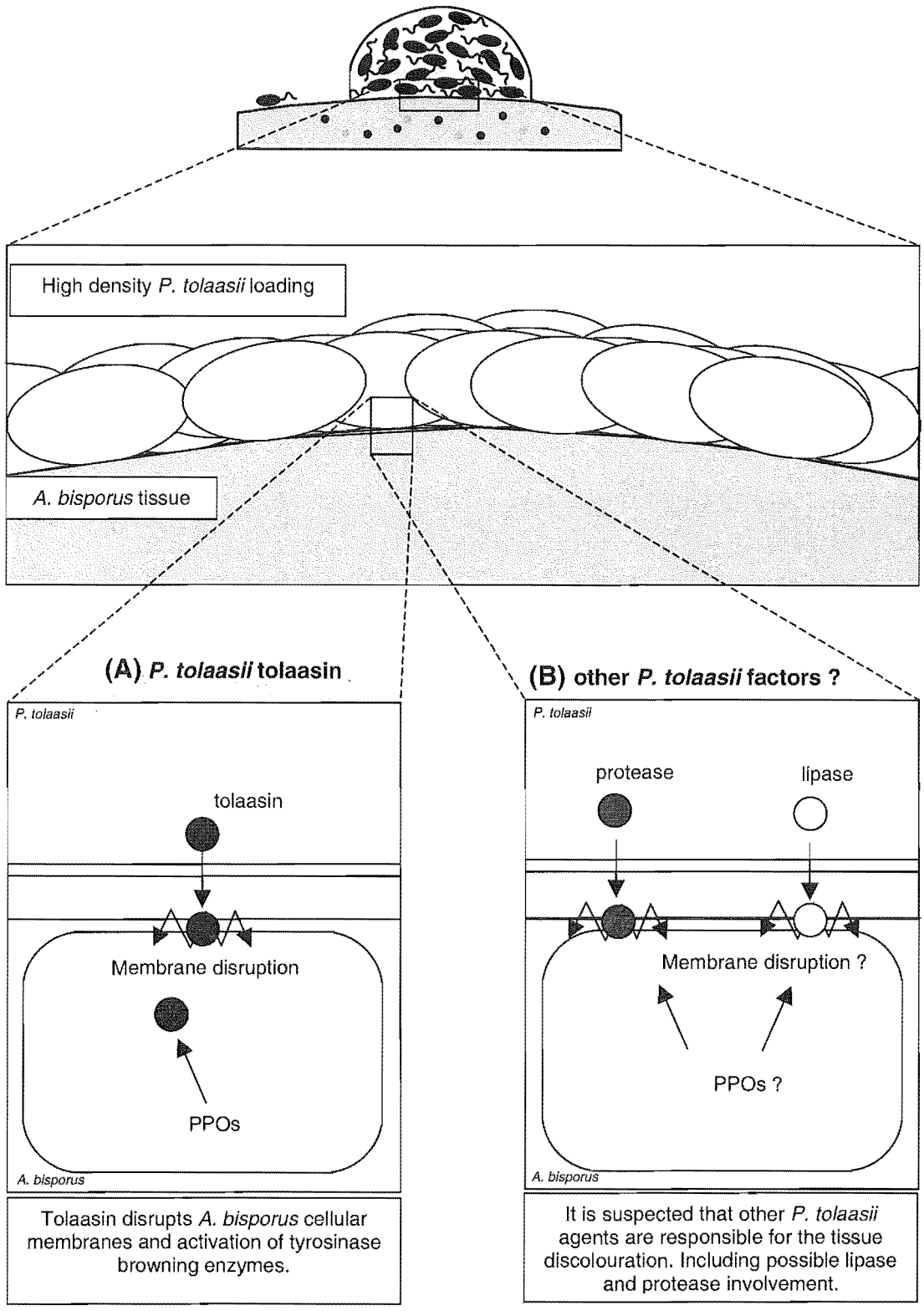
Model 2 Common to blotch discolouration of growing *A. bisporus* are unmonitored abiotic factors involved with water condensation on the mushroom cap. (A) Water droplets form on a mushroom cap during over-watering and/or incorrect humidity monitoring. (B) If correct abiotic environmental conditions are implemented, evaporation of water droplets will occur (Bi) which results in non-blotch affected mushrooms (Bii and Biii). (C) If water droplets remain on the mushroom tissue, the high concentration of nutrients found in mushroom tissue will leach by osmosis into the water droplet (Ci). This then provides a nutrient rich medium for exponential bacterial growth (Cii and Ciii) and high bacterial numbers reach a threshold and blotch discolouration forms (Civ).

7.3 Model 3 *P. tolaasii* blotch formation

How does a bacterial threshold on the mushroom cap cause blotch disease? Of the BCOs described currently in the literature, *P. tolaasii* is the most extensively characterised[1]. The primary causal agent of *P. tolaasii* blotch is tolaasin[2], an extracellularly produced lipodepsipeptide that causes membrane disruption and activation of polyphenol oxidase (PPO) enzymes[3] that result in brown discolouration of *A. bisporus* mushroom tissue (Model 3-A). However, studies have also acknowledged that apart from tolaasin, there are other factors that induce the brown discolouration caused by *P. tolaasii*; including possible factors such as protease and lipase production[4] (Model 3-B), but their mode of action and direct involvement have not yet been established.

As this study showed that lipodepsipeptide production was not the primary determinant of blotch discolouration by the BCOs[5], it was hypothesized that another extracellular compound(s) may be the common BIF(s) amongst BCOs.

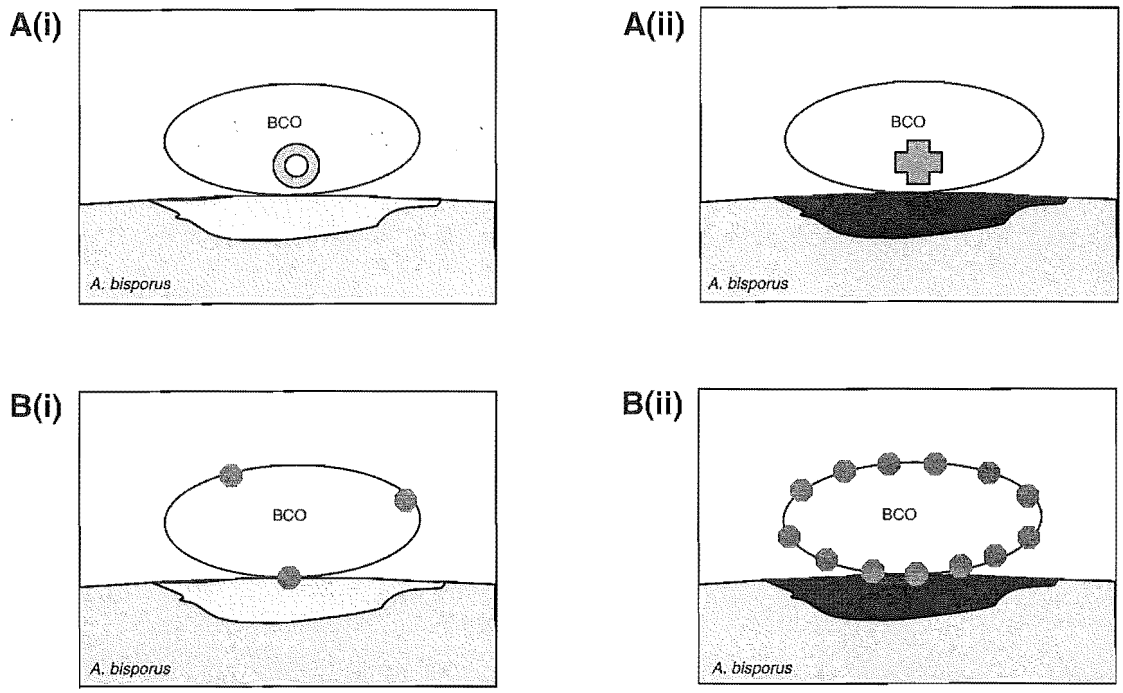
[1] Chapters 1.5, Chapter 2.3.1 and Chapter 3.1
[2] Chapter 3.3
[3] Chapter 1.2
[4] Chapter 3.4, Chapter 4.4.3 and 4.4.4
[5] With exception of NZ027, NZ032 (Chapter 2.11) and NZI7 (Chapter 3).



Model 3 (A) In *P. tolaasii,* the primary causal agent of blotch formation is the production of the extracellular lipodepsipeptide, tolaasin, that causes cellular disruption in the membrane of *A. bisporus*, causing pitting and activation of major PPO enzymes involved in browning of *A. bisporus* tissue such as tyrosinase (B). Other factors are suspected to be involved in *P. tolaasii* browning and may include extracellular enzymes such as protease and lipases.

7.4 Model 4 Blotch inducing factors (BIFs) of blotch discolouration

Not all pseudomonads in the mushroom environment are able to cause blotch discolourations of *A. bisporus* tissue and different pseudomonad species that produce blotch, may produce different discolourations[1]. Taking these facts into account, it is clear that those pseudomonads able to produce blotch (BCOs), must have 'something' that induces the discolouration of *A. bisporus* mushroom tissue (BIF(s) of BCOs). Because not all bacteria induce blotch, it is likely that BIFs are extracellularly produced compounds expressed only by BCOs. Furthermore, given that different BCOs can cause blotch of variable discolouration[2] is suggestive that either: different BCOs have different BIF(s) that cause different blotch discolourations (Model 4-A); and/or that different amounts of BIFs are required to cause different *A. bisporus* blotch discolourations (Model 4-B).



Model 4 Different BCOs can cause different blotch discolourations. Two scenarios that could explain this observation is: (A) Different BIFs cause different amount of mushroom discolouration; or (B) different BCOs have different amounts of BIF(s) where cells with low BIF concentration (Bi) elicit discolouration of a low scale and cells with a high BIF concentration (Bii) produce a dark *A. bisporus* discolouration.

[1] Chapter 2
[2] Table 2-2

7.5 Model 5 BIF(s) are concentration dependant

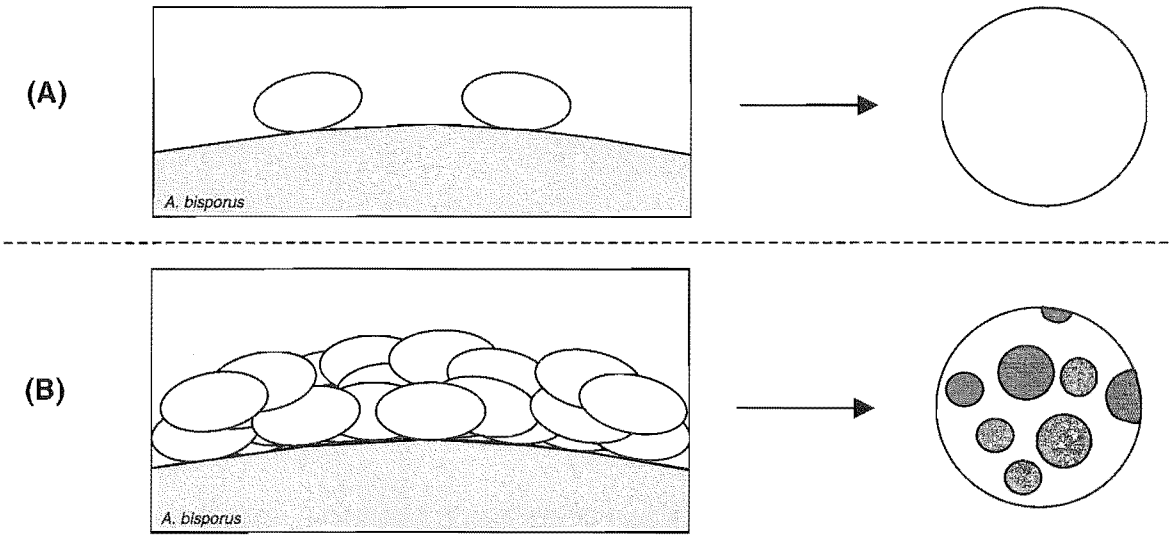
BIF(s) are not specific to mushroom isolated pseudomonads

The observation that pseudomonads from another environmental niche (a milk production factory) were able to induce blotch discolouration as efficiently as those pseudomonads from the mushroom niche[1], gave rise to the hypothesis that blotch may not be an acquired pathogenicity adaptation by pseudomonads within the mushroom farm niche, but simply a defense mechanism by *A. bisporus* tissue in response to the establishment of opportunistic bacteria reaching a cell-density threshold on the mushroom cap surface.

BIF(s) are required at high concentrations to induce blotch discolourations

BIFs may be present amongst many different pseudomonad species from varying environmental niches, but what they have in common is that at cell-density thresholds they are able to elicit an *A. bisporus* response that is manifested as tissue discolouration, *i.e.* blotch[2]. If BCOs produce BIF(s) from individual cells (*i.e.* a constitutive mechanism not regulated by environmental situations), the following model is proposed in which *A. bisporus* blotch discolouration does not occur at low BCO cell-density (Model 5-A) but when a cell-density threshold is present, high concentrations of BIFs result in a *A. bisporus* discolouration response. (Model 5-B).

[1] Chapter 2.14 and discussed in Chapter 2.14.15.
[2] Chapter 1.5.6



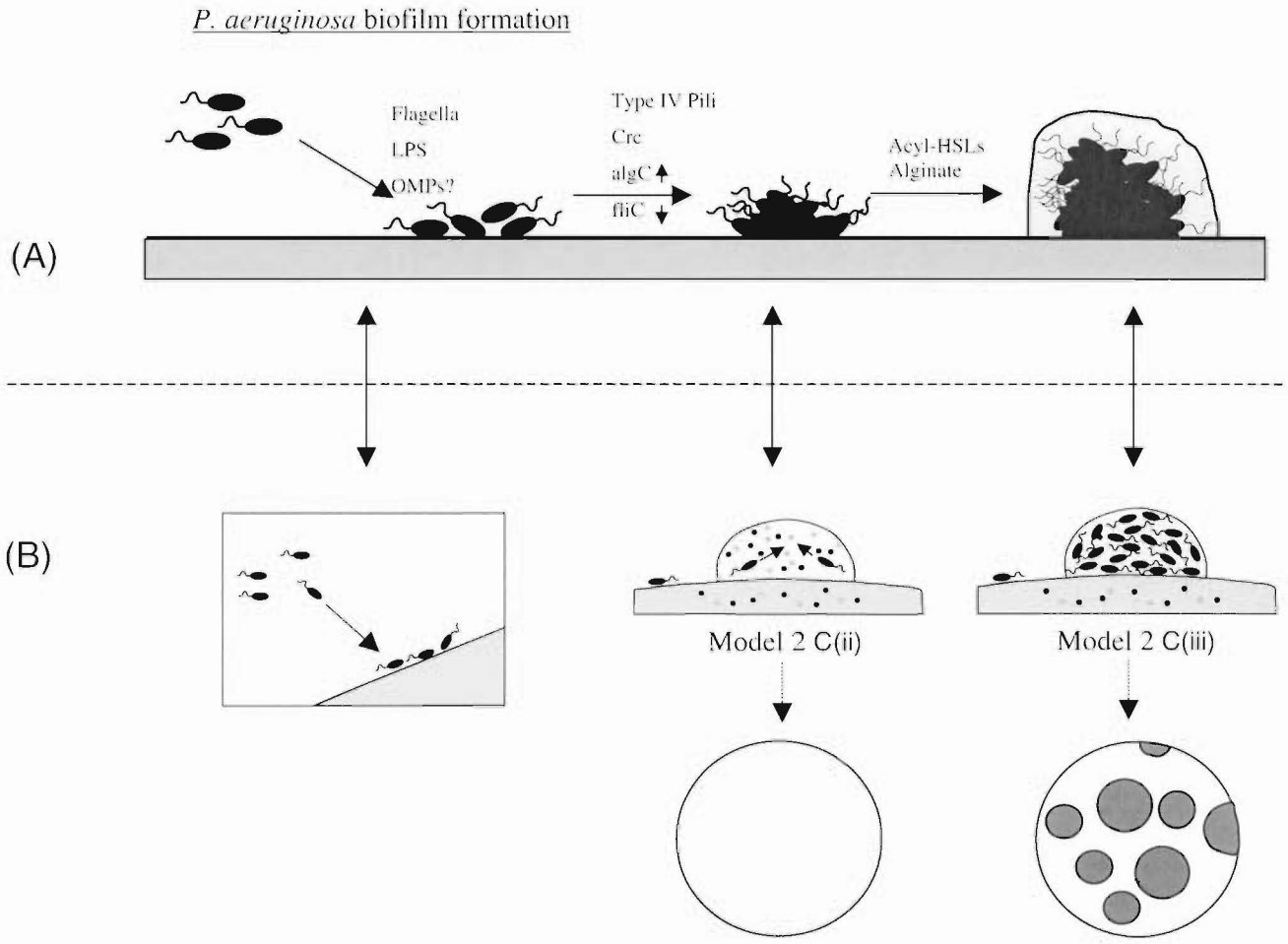
Model 5 Many different pseudomonads have BIFs that may cause *A. bisporus* tissue discolourations. (A) Low cell-densities do not provide a high enough concentration of these BIFs to cause tissue discolouration. (B) If conditions favour opportunistic bacterial growth to a cell-density threshold, BIFs are then at a concentration sufficient enough to elicit a response by *A. bisporus* causing a blotch discolouration.

7.6 Model 6 Cell-to-cell communication in biofilms provide BIFs

A further model to explain why high bacterial cell-densities are required to cause blotch discolourations is that cellular production of the BIF(s) may be induced at these high bacterial thresholds. This would be applicable where a threshold of bacteria is required to form a mature biofilm. Within this mature biofilm, cells coordinate production of secondary compounds by means of intercellular signaling (quorum-sensing)[1]. Thus, secondary compounds (such as a BIF) may not be produced until cells aggregate into a mature biofilm, that is, a high bacterial density threshold.

Within this study, the only putative pathogenicity determinant found to be consistently observed amongst the 33 BCOs characterised was that of biofilm formation[2]. Biofilm formation requires surface and nutritional requirements for the establishment and cellular propagation of a vast number of cells in which the biofilm community exists. Biofilm formation (Model 6-A) best fits into the present understanding of the epidemiology of blotch formation (Model 2) in that the establishment of bacterial thresholds are required for blotch discolouration and therefore, the stages of biofilm formation can be correlated with stages of blotch disease establishment to generate (Model 6-B). Although it was established that: (i) non-blotch inducing isolates from Chapter 2 had the ability to form biofilms[3]; and (ii) one NZ103 biofilm deficient mutant remained able to cause wildtype blotch discolouration[4], it cannot be discounted that a component(s) of biofilm formation may be involved in blotch discolouration.

[1] Chapter 4.3.3
[2] Table 4-1 and discussed in Chapter 4.9.1
[3] Table 2-2
[4] Isolate 103G4 Table 5-1

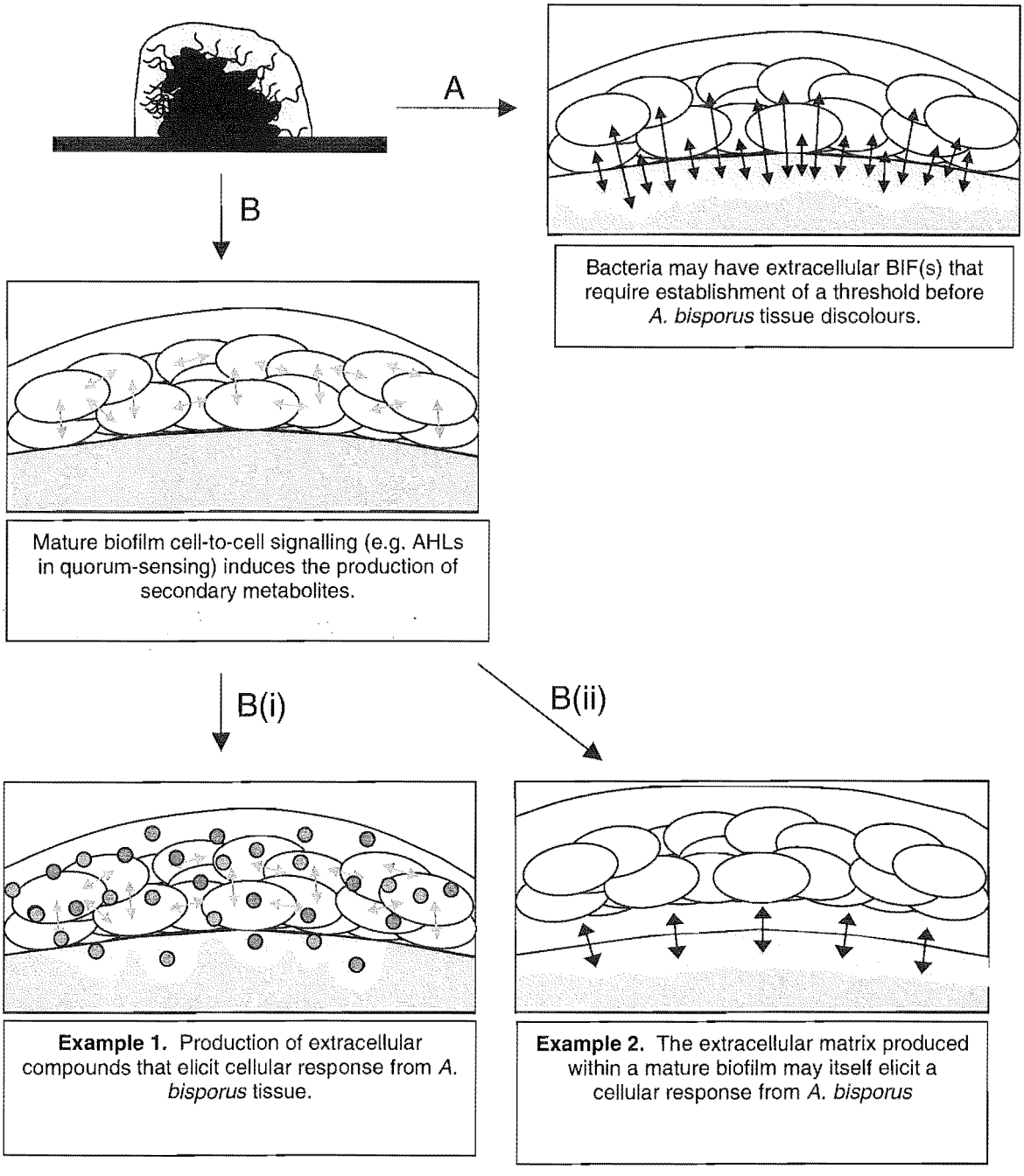


Model 6 (A) *P. aeruginosa* biofilm formation model (Davey and O'Toole, 2000) in which planktonic cells migrate and attach to a given surface, aggregate, and then through coordinated cellular contact, produce a mature biofilm. (B) Speculative correlation of the biofilm formation model with important stages of *A. bisporus* blotch discolouration described in Model 2.

7.7 Model 7 How do biofilms provide a BIF(s)

Biofilm formation often involves intracellular communication (quorum-sensing) that is responsible for the production of secondary compounds required for biofilm organization[1]. Biofilm formation on a mushroom surface may simply provide a situation where a high number of bacterial cells are present with each cell having a BIF(s) and when the cell-density reaches a threshold, the concentration of BIF(s) induce disease (Model 5, Model 7-A). However, a different model may be proposed in which secondary compounds mediated by quorum-sensing and upregulation of biochemical pathways required for biofilm formation, may produce extracellular components that within themselves become the BIF(s). Such BIF(s) produced in response to quorum-sensing could include: (Model 7-B(i)) extracellular BIF(s) that are translocated across the bacterial cell wall and interact with the *A. bisporus* tissue membrane; or (Model 7-B(ii)) the BIF(s) may be components of the mature biofilm matrix (e.g. EPS) and this may elicit a cellular response within *A. bisporus* tissue.

[1] Chapter 4.2.3 and Chapter 4.3.3

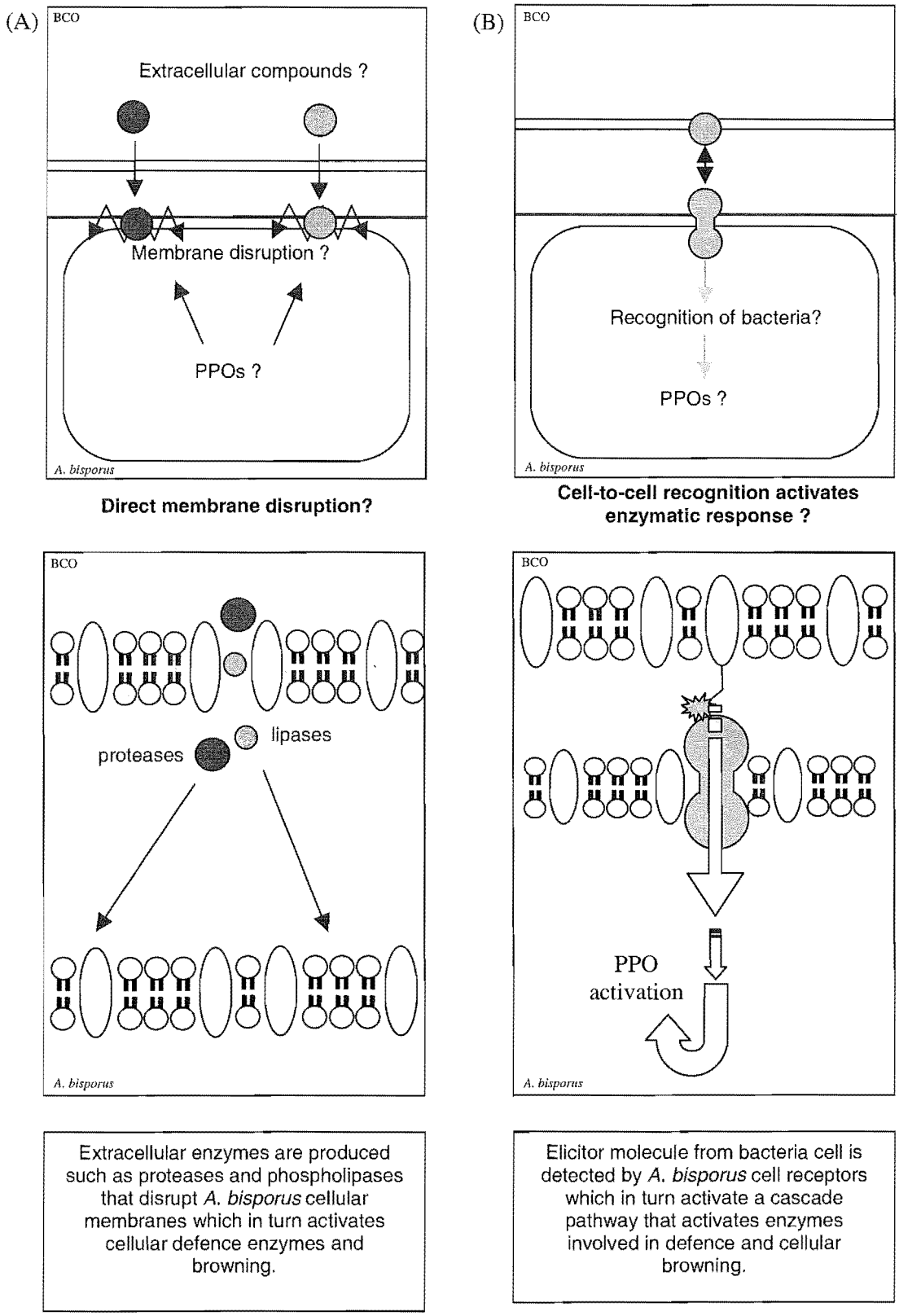


Model 7 Possible mechanisms in which mature biofilms may induce blotch discolouration of *A. bisporus* tissue: (A) Biofilms consist of a number of cells that may simply provide the cell-density threshold of BIFs required to cause blotch discolouration (as described in Model 5); or (B) Biofilm formation may involve cell-to-cell communication (quorum-sensing) in which cells coordinate to produce secondary compounds required for mature biofilm formation. These extracellular compounds may be (B(i)) compounds that directly interact with *A. bisporus* cellular membranes; or (B(ii)) compounds that are present in the biofilm matrix (e.g. EPS) that elicit a cellular response in *A. bisporus* causing the discolouration process.

7.8 Model 8 *A. bisporus* response to BIFs

The relationship between a bacteria and its host is dynamic, since each modifies the activities and functions of the other. It is evident that BCOs produce BIF(s) that elicit a response by *A. bisporus* that results in tissue discolouration. Some BCOs generate a direct response through disruption of *A. bisporus* cellular components (such as cell membrane disturbance by *P. tolaasii* tolaasin). However, given the continuing research into enzyme production in *A. bisporus* responsible for tissue discolouration and defence mechanisms[1], it is highly likely that blotch discolourations are a result of *A. bisporus* enzymatic reactions in response to bacterial BIFs. BCOs may not deliberately produce extracellular compounds in a classical pathogenic response to the presence of a host, but rather, *A. bisporus* may simply detect the presence of 'potentially threatening' non-host cells (*i.e.* bacterial BIFs) on its cellular surface (*i.e.* the mushroom cap). Bacteria will multiply given the favourable environment provided by water droplets on mushroom caps (Model 2) and therefore, this bacterial threshold contains BIFs that most likely activate some type *A. bisporus* cellular recognition process. Therefore, one can speculate that the origin of these BIFs are possibly: (Model 8-A) an extracellular compound that is produced and exported across the bacterial cell wall into the surrounding medium that comes in contact with fungal cells; or (Model 8-B) an extracellular component of the bacterial cell wall that is recognised by the fungal cell. These two scenarios encompass many biochemical and physiological occurrences, but it is likely that either of these putative BIFs induce a mediated response from *A. bisporus* mushroom tissue that results in blotch discolourations.

[1] Chapter 1.2

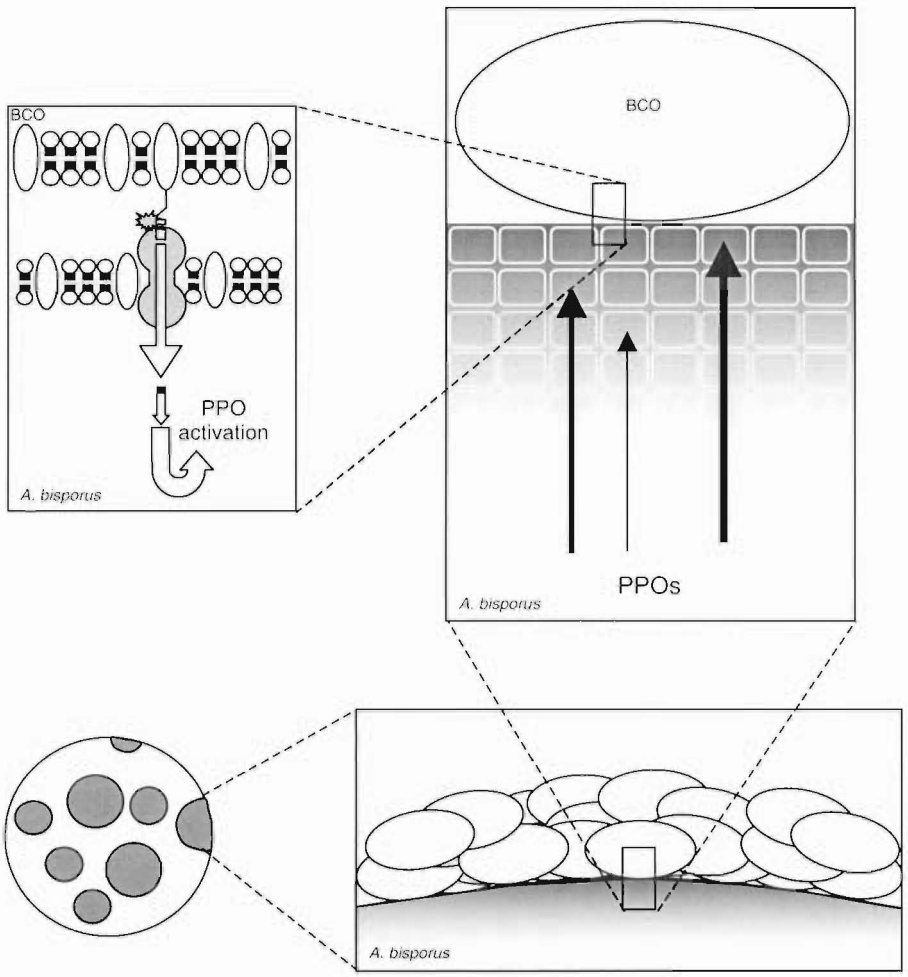


Model 8 Two broad scenarios are proposed to encompass the likely BIFs of *A. bisporus* mushroom tissue: (A) the elicitor is an extracellular compound excreted from the BCO cell wall that interacts with the *A. bisporus* cell membrane; and/or (B) BIFs are extracellular components of the cell wall of BCOs. Both scenarios are likely to induce receptor-mediated response from *A. bisporus*

7.9 Model 9 BIFs induce *A. bisporus* enzymatic defence mechanisms

Given the scenario that BIFs induce a response from *A. bisporus* mushroom tissue (Model 8) resulting in blotch discolourations, the most probable intracellular regulation would be the activation of PPOs (which includes tyrosinase)[1] as a defence mechanism to the potential threat of foreign microbial colonisation. Such enzymes could be secreted to *A. bisporus* tissues that are in contact with the colonised bacterial cells to provide a protection barrier between itself and potentially invading cells. PPOs cause production of the pigmented compounds, such as melanin[2], and therefore blotch discolouration of tissue is likely due to the production of these enzymes. Furthermore, the type and concentration of these PPOs would give a possible explanation as to why different degrees of blotch discolouration is observed amongst BCOs.

[1] Chapter 1.2
[2] Chapter 1.2.1

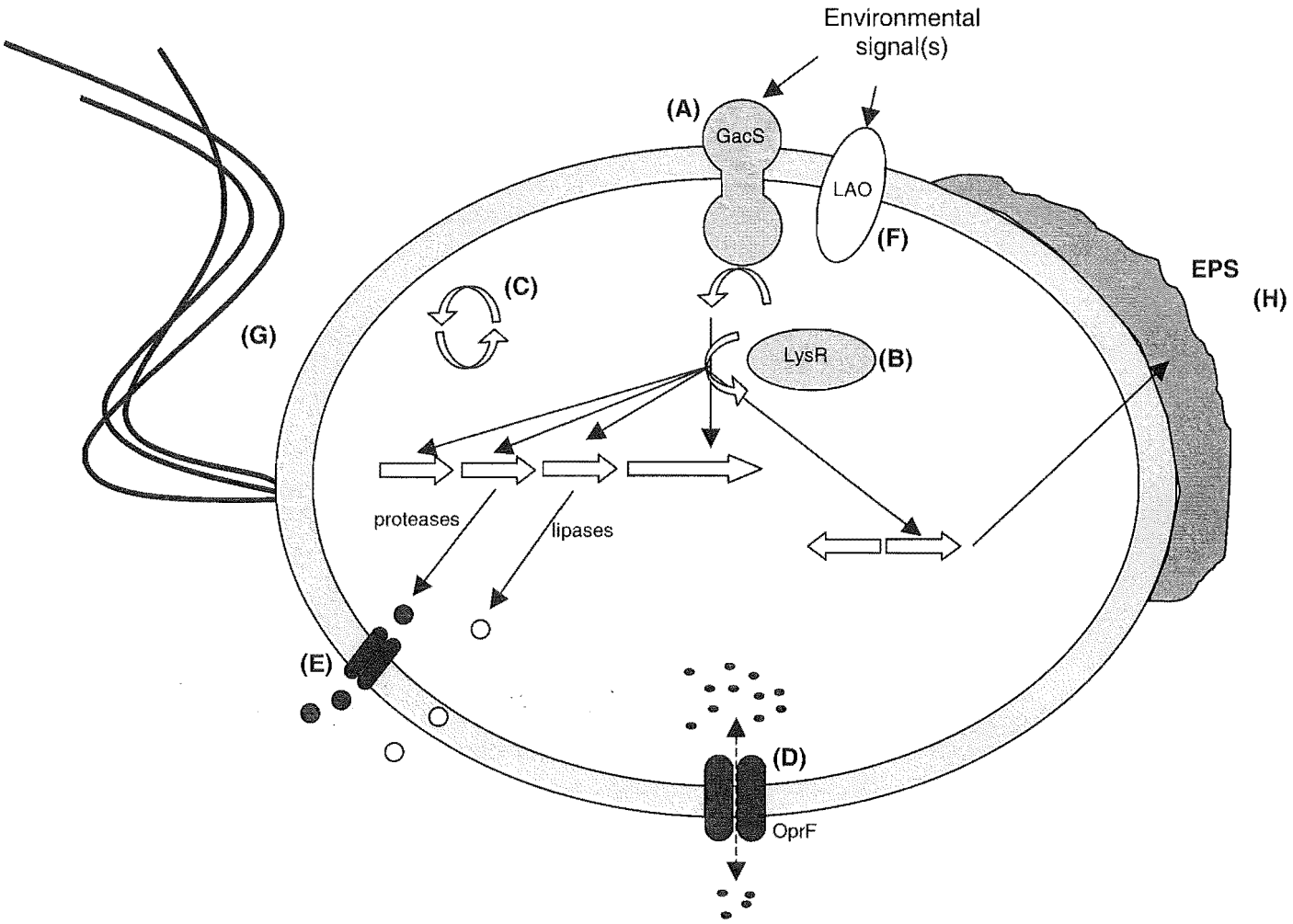


Model 9 In response to the BIF(s) produced by colonising BCOs, *A. bisporus* may elicit a defence response within mushroom tissue that results in the production of enzymes that produce oxidation of tissue in contact with the bacterial cells. This enzymatic process could render a barrier between the colonised bacteria and the fungal tissue. Bacterial blotch may not be a result of pathogenicity, rather a natural defence system in *A. bisporus* to prevent colonisation of other microorganisms.

7.10 Model 10 Bacterial cell model of NZ103 given Tn5 mutant analysis

The mutagenesis screen of NZ103 performed within this study did reveal mutants deficient in the genetic regulation of certain pathways that ultimately had effect on the degree of blotch discolouration[1]. Because of the complexity of genetic regulation of particular phenotypes, it would be unwise to assign the identified NZ103 disrupted genes that result in blotch reduced phenotypes as BIFs. Because mutants were not functional characterised within this study, it is more appropriate to describe these genes and gene functions as important cellular regulatory factors that contribute to the bacterial colonisation of NZ103 on *A. bisporus* mushroom tissue. These genes and gene products are schematically depicted in Model 10.

[1] Table 5-1



Model 10 Bacterial cell model of NZ103 depicting predicted involvement of genes and gene products in wildtype regulation for colonisation of *A. bisporus*. Depicted are homologs to: (A) GacS/RtpA involved in two-component regulatory systems; (B) LysR transcriptional regulator; (C) 2-hydroxyacid dehydrogenase involved in cellular metabolism; (D) OprF outer membrane protein involved in osmoregulation and cell adhesion; (E) AprE/EprE involved in metalloprotease secretion; (F) LAO extracellular binding protein that may act as a chemoreceptor, transport systems and/or initiate signal transduction pathways; (G) Flagella are involved in motility; and (H) EPS is likely an important factor in biofilm formation.

FINAL CONCLUSION

This thesis originally set out to characterise the causal organism of 'ginger blotch' disease of *A. bisporus* within New Zealand mushroom farms. Typically, a given disease is described as being caused by a single species of organism and that organism is described as a pathogen that exhibits defined pathogenicity determinants. However, 'ginger blotch' was found to be caused by a number of different *Pseudomonas* species, and furthermore, these *Pseudomonas* species had no common pathogenicity determinant(s) (tested within this thesis) that were empirically shown to cause blotch discolourations. Because different *Pseudomonas* species could elicit different *A. bisporus* discolourations, it was established that blotch-inducing factors (BIFs) were involved in the discolouration process. Recognition of these BIFs by *A. bisporus* tissue is suggested to induce a defense mechanism that involves the production of polyphenol oxidase (PPO) enzymes that provide a harsh environment to prevent further colonisation by BCOs. A consequence of this defense mechanism is tissue discolouration, that is the manifestation of the phenotype commonly viewed as blotch discolourations. Furthermore, because different species induced different blotch discolourations, it is suggested that the degree of discolouration is a result of the degree of enzymatic activation of the PPOs in localised *A. bisporus* tissue. Therefore, 'ginger blotch' and other blotch diseases may not be the result of pathogenicity determinants acquired by pseudomonads in the mushroom environment to provide a selective advantage, but rather, blotch may be simply a fungal defense response to the detection of opportunistic foreign bacterial cells growing in a nutritionally rich environment.

Chapter 8 FUTURE DIRECTIONS

As the results of this thesis have contributed to the knowledge of bacteria blotch, it is just as important to consider a continuation of research based on the findings in this thesis to clarify current understanding and to explore new potentially rewarding avenues. Presented in the following sections are suggestions as to future research that would further the understanding of bacterial/fungal interactions of pseudomonads isolated in this study and substantiate or otherwise, the models proposed in this study.

8.1 Population studies of pseudomonads causing blotch

It is felt that the number and diversity of the pseudomonads isolated during this thesis form more than an adequate foundation for future studies. Although, one area that would contribute to the further understanding the population dynamics of BCOs, would be to genotypically characterise the distribution and frequency of BCOs to determine the predominant blotch causative organism within New Zealand and/or on any given farm. Such information would allow more applied studies to determine the epidemiology of the prominent BCOs with a targeted outcome of implementing proper farm management strategies for its control.

One proposed interesting applied epidemiology study that would provide strong foundation of microbial blotch and polymicrobial effects would be to analyse populations of bacteria on *A. bisporus* exhibiting blotch compared with non-blotched mushrooms to determine whether blotch is associated with higher numbers of BCOs or a different populations. This would lead nicely into polymicrobial effects which are undoubtedly involved in the establishment of blotch. Poly microbial activity involves the interaction of microorganisms within a given niche and these interactions will undoubtedly have implications on the success or otherwise of a given pathogen expression its pathogenic phenotype. Many antagonists of pathogens exist which are shown to adversely affect the growth of other microorganisms and this is well studied within the area of biocontrol agents. Conversely, many organisms have been shown to beneficially exert an affect on other microorganisms. Therefore, it cannot be discounted that the 33 BCOs within Chapter 2 of this thesis may not exert an effect on the mushroom and cause blotch under with poly microbial interactions. It is noted that the 33 BCOs were identified as causing blotch by growing pure cultures and inoculating mushroom cubes which deliberately have the cap and potential contaminants removed. Thus, these organisms are exhibiting a blotch phenotype without the interaction of other microorganisms that may in fact inhibit their growth and prevent the BCOs from reaching the well-established threshold for disease induction. Further investigation of polymicrobial effects will greatly aid the epidemiological understanding of bacterial blotch.

8.2 Further characterisation of NZI7 LDP

The identification of *Pseudomonas* NZI7 that phenotypically resembled *P. tolaasii* in blotch discolouration and lipodepsipeptide (LDP) production was an interesting observation that impacts on the validity of long-established *in vitro* methods of determining *P. tolaasii* identity. Furthermore, it raises the question as to gene acquisition amongst pseudomonads in the mushroom farm[1]. NZI7 genotypically is similar to *P. syringae*, but the LDP resembles to *P. tolaasii* tolaasin. To substantiate the possibility that the LDP in NZI7 may have originated from *P. tolaasii*, further characterisation of the NZI7-LDP is required. This should include full characterisation of the genetic region(s) required for NZI7-LDP production and comparison to *P. tolaasii* tolaasin gene cluster (Rainey, *et al.*, 1993), functional analysis of its role in membrane disruption should also be determined. Sequence comparison to *P. syringae* syringomycin genes should also take place as NZI7-LDP and syringomycin have high similarity.

8.3 NZ103 mini-Tn*5*km*lacZ*2 mutants

The limitations of screening only 5000 mutants and selecting for a particular phenotype(s) based on assays with defined parameters, may have biased and limited the number of mutants obtained in this study. Nonetheless, 45 mutants (Table 5-1) were generated that exhibited a diverse range of phenotypes and these cryogenically stored mutants will provide a valuable resource for future investigations of bacterial/fungal interactions and blotch discolouration of *A. bisporus*, such as those proposed below.

8.3.1 GacS/GacA/RtpA homologs in 103G3

A holistic view of the results obtained in this thesis establishes the prominent fact that blotch formation is multifactor with many complex interactions between environmental signals and expression of genetic regulatory pathways. This was confirmed by the identification of homology in the mutant 103G3 that showed mini-Tn*5*km*lacZ*2 disruption was to nucleotide sequence that showed translation similarity to the two-component regulatory systems of GacS/GacA (Heeb and Haas, 2001). Furthermore, this system was shown to have similarity to a previously identified RtpA mediated two-component regulatory protein in *P. tolaasii* (Murata, *et al.*, 1998). The mini-Tn*5*km*lacZ*2 insertion caused 103G3 to have more than one putative pathogenicity determinant to be disrupted, suggesting that these phenotypes are: (i) under the global control of the GacS/GacA two-component regulatory system; and (ii) that GacS positively regulates their phenotypic expression.

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[1] Presented in Section 8.5 is a discussion of the possible role(s) of horizontal transfer in the mushroom farm.

Two-component regulatory proteins are widely implemented in the regulation of multiple gene functions as a result of a signal from an external cellular source. A full characterisation of the GacS/GacA homologue in 103G3 (103G3-GacS) would provide many beneficial outcomes, including: (A) enable comparison of 103G3-GacS similarly to the previously reported two-component regulatory systems in *P. tolaasii*, *i.e.* RtpA (Murata, *et al.*, 1998) and PheN (Grewal, *et al.*, 1995); (B) enable functional studies of 103G3-GacS by development a genetic reporter system to qualitatively determine when 103G3-GacS regulation occurs and what critical environmental signals are required for NZ103 genetic pathways; and (C) 2D-gel electrophoresis could be carried out to determine the number of proteins regulated by 103G3-GacS and subsequent purification N-terminus sequencing could provide information of the functionality of these regulated products. Addressing such areas would provide a 2-fold outcome: (i) a fundamental understanding of a component of bacterial environment regulation; and (ii) elucidation of this will determine the importance of environmental signals in the phenotypic expression of blotch discolouration and bacterial colonisation would enable an applied focus on developing bacterial blotch control strategies. Furthermore, a limited number of PPDs were analysed within this study and it is likely that other biochemical and physiological deficiencies have occurred in 103G3 due to mini-Tn*5*km*lac*Z2 insertion. Identification of these may lead to new factors being identified determinants in blotch disease (BIFs).

8.3.2 103F5 OprF homolog unable to grow on MJA

Previously discussed in Chapters 5.9 and 6.3.7, was the special interest placed in the mutant 103F5 that was unable to grow on MJA because of a disruption to a genetic region with high homology to the major outer membrane protein, OprF. OprF is required for cell growth in low-osmolarity medium and for the maintenance of cell shape (Gotoh, *et al.*, 1989, Woodruff and Hancock, 1989) and has further been described as a bi-functional protein having porin activity, forming small water-filled channels (Woodruff and Hancock, 1988). Furthermore, an OprF-like protein has recently been suggested to be involved in adhesion between a *P. fluorescens* strain and plant roots (De Mot and Vanderleyden, 1991, De Mot, *et al.*, 1992, De Mot, *et al.*, 1994). These combined observations provide an interesting background in which gaining further understanding of the role that outer membrane proteins play in NZ103 would greatly add to the knowledge base of bacterial/fungal interactions.

103F5 is currently the focus of investigation to further characterise the genetic region(s) disrupted by mini-Tn*5*km*lac*Z2 and this has been initiated with the recovery of cosmids from a 103F5 cosmid library by probing with regions of nucleotide sequence generated within this study. Characterisation of further genetic regions will enable subsequent functional analyses of the OprF in NZ103 by using *trans*-complementation with *recA* deficient NZ103 strains[1]. Expression studies of OprF will be carried out using reporter genes to determine gene expression under various environmental situations with

[1] The creation progress of creating NZ103 strains deficient in recA is discussed in the following section 8.4.

focus on osmotic variation. It is a hypothesis that osmoregulation is an important factor contributing to bacterial colonisation of NZ103 and OprF is involved in this function.

8.3.3 Biofilm defective NZ103 strains

Results obtained from the abiotic biofilm assays were not utilised as comprehensively as they could have been. An initial focus of Chapter 4 was to attempt to characterise what PPDs may cause *A. bisporus* blotch discolourations and as a result of this exploratory approach, attention and time was diffused over a number of different phenotypes. However, it remains a major conclusion from this thesis that biofilm formation is an important physiological function of BCOs involved in the colonisation and/or eliciting blotch discolourations of *A. bisporus*. Many theses have focused solely upon studying biofilm formation and therefore, many of the mutants obtained with biofilm deficiencies warrant future investigation. These include: (1) The observation that all 14 biofilm mutants were shown to have normal motility activity (Table 5-1) and conversely, nine NZ103 mutants with reduced or deficient motility showed comparable biofilms to the wildtype (Table 5-1). These observations would suggest that motility is not a critical factor in the formation of an abiotic biofilm given the conditions outlined in this study. Therefore, it would be beneficial to determine what other factors contribute to NZ103 biofilm formation, for example, confirm whether EPS has a major involvement in biofilms such as was suggested by TEM studies; (2) As biofilm mutant 103G3 was identified as having a mini-Tn*5*km*lac*Z2 disruption in a two-component regulatory system homologous to GacS/GacA/rtpA, 103G3 warrants further investigation to understand what environmental signal(s) this system is regulated upon and what other genetic pathways are concurrently regulated that result in the biofilm deficient phenotype; (3) It would be interesting to fully characterise NZB5++ to assess the possible involvement of cell-to-cell communication (such as the production of AHLs involved in quorum-sensing) in NZ103 biofilm formation. Discovery of a coordinated cell-to-cell communication signaling process may link quorum sensing and two-component regulatory protein such as GacS seen in 103G3 described above. Characterisation of such processes involved in NZ103 biofilm formation would enable comparison to previously described bacterial species in order to establish the degree of similarity (or otherwise) amongst biofilm formation in different environmental niches and elucidate special biofilm regulation in a bacteria/fungal interaction.

8.3.4 Biofilm defective *P. tolaasii* strains

P. tolaasii biofilm formation has not been reported in the literature and therefore the result that *P. tolaasii* produced abiotic biofilms in Chapter 4.7.1 was a novel observation. What was not described in the text of this chapter was that *P. tolaasii* NCPPB2192^T was concurrently mutagenised with mini-Tn*5*km*lac*Z2 during this study and screened for biofilm loss only. From a pool of 3000 mutants, 38

mutants had altered biofilm phenotypes[1] (36 biofilm deficient and two biofilm enhanced, Appendix IV(i)). Given the prominence of *P. tolaasii* in the literature, it would be beneficial to characterise these biofilm defective mutants, (similar to what was performed for NZ103 in Chapter 6 – but in a focused study purely on biofilm formation) and compare the findings to the genetic information of NZ103 and other pseudomonads able to form biofilms. The observation of biofilm-enhanced mutants also suggests that quorum sensing may be a regulatory mechanism in the phenotypic expression of *P. tolaasii* NCPPB2192. Because of the depth of *P. tolaasii* investigations, it is considered that a wider interest would be generated in reporting mechanisms involved in the biofilm formation of the type strain, *P. tolaasii* NCPPB2192, rather than NZ103; which relatively, is an unknown organism.

8.4 RecA mutations

In order to develop effective strategies for functionality studies of the mini-Tn*5*km*lac*Z2 transposon mutants described above, it is proposed that *recA* deficient strains of NZ103 be created. At the stage of submission of this thesis, progress has been made into the creation of such strains to enable *trans*-complementation analysis of OprF in NZ103. Strains deficient in RecA (the protein produced by the *recA* gene) will greatly aid in further characterisation of the cellular physiological function of selected NZ103 mutants in relation to NZ103 wildtype activity.

Complementation studies of transposon mutants

Much of the current knowledge of the bacterial molecular mechanisms have been elucidated using the study of mutants, such as those derived with mini-Tn*5*km*lac*Z2 in Chapter 5. Once these mutants are created, it is desirable to confirm that the mutation under investigation is indeed the cause of the altered phenotype. Many approaches have been employed for such confirmation, including: transduction; *trans*-complementation; allele exchange, and bioinfomatics.

Trans-complementation is a method that involves the isolation of the complementary DNA region from the parental strain to the mutation and then allows assessment of this wild type DNA to restore the wild type phenotypes. A factor of *trans*-complementation is that the original mutated DNA region remains in the mutant and the introduced wild-type DNA is present in a stable vector. As this method does not facilitate the replacement of the mutated region, it requires the complementing sequence to be sufficiently expressed in the vector system to restore the desired wild-type phenotype. Thus, *trans* complementation has the additional benefit of providing information regarding the presence of intact genes or operons within the complementing sequence.

Allele exchange differs from *trans*-complementation in that the mutated sequence is replaced by the wild type allele. Allele exchange is dependant on the presence of DNA sequence from both sides of the mutation being present in the wild type allele and recombination will occur in the presence of

[1] Note that these mutants have been cryogenically stored for future research.

RecA to replace the mutated region. In the case of transposon mutagenesis, recombination of wild type DNA replacing the mutated DNA region can be easily screened by the loss of antibiotic resistance associated with the transposon. If following recombination events, the mutant no longer expresses the mutant phenotype, (*i.e.* exhibits the wild type phenotype) confidence can be placed in the original mutation being responsible for the observed phenotype. Furthermore, allele exchange has the added benefit in that it can be used in the opposite fashion as above, that is, the region of chromosome containing the mutation can be isolated and recombined in a similar fashion back into the wild type parental strain. Subsequently, if the phenotype of the newly created mutant is identical to that of the original mutant, verification can be sort that the mutation is responsible to that particular phenotype.

Complementation in *P. putida NZ103*

Given the potential benefits of using *trans*-complementation, the development of a system that would permit *trans*-complementation was undertaken. Although some cloning vectors that are stable in *Pseudomonas* spp. are available (Heeb, *et al.*, 2000), their use for *trans*-complementation can be limiting as previous studies have shown vector instability and the creation of a *rec*A defective pseudomonad strain increases capabilities of studies in *trans* (Silby and Mahanty, 2000).

The RecA protein

RecA has long been recognised for its involvement in homologous recombination between incoming DNA and the recipient genome (Clark and Margulies, 1965, Kowalczykowski, *et al.*, 1994), recombinational DNA repair and the SOS response (Miller and Kokjohn, 1990). Additionally, RecA has also been identified as important in the homologous recombination and DNA repair in pseudomonads, including: *P. fluorescens* (De Mot, *et al.*, 1993); *P. putida* (Luo, *et al.*, 1993); *P. aeruginosa* (Sano and Kageyama, 1987); and *P. aureofaciens* (Silby and Mahanty, 2000). Furthermore, a specific study on *P. tolaasii* was carried out and showed that phenotypic switching of *P. tolaasii* NCPPB 1116 from smooth to rough was dependent on *recA* whereas *recA* is not required for the rough to smooth transition (Sinha, *et al.*, 2000).

Two major objectives can be outlined for (i) the creation of *recA* deficient strain(s) of NZ103; and (ii) determining the usefulness of these strains in *trans*-complementation systems for functional analyses of NZ103 mutants. To achieve these objectives, several aims are defined:

1. Recover a *recA*+ cosmid from the NZ103 library.
2. Demonstrate that NZ103 *recA* can restore *recA*-dependent functions to *recA* mutants of *E. coli.*
3. Determine the nucleotide sequence of the NZ103 *recA* gene.
4. Introduce an antibiotic cassette to disrupt NZ103 *recA* in a cosmid and homologously recombine the mutated *recA* into the NZ103 genome.
5. Assess the effect of disrupting NZ103 *recA* on introduced cosmid stability and recombination frequencies.

6. Develop a system that would allow *recA* to be mutated in selected Tn*5* generated NZ103 mutants from Chapter 5 and Chapter 6.

At submission of this thesis, the isolation and subcloning of the NZ103 *recA* has been achieved by creating genomic libraries of NZ103 in pSK- using a single enzyme to digest separate libraries (KpnI, XbaI, and *Pst*I). These genomic libraries of NZ103 were electroporated into *E. coli* DH10B (*recA*$^-$) and were plated on LB plates supplemented with ampicillin (selection of pSK-) and MMS (to select for functional copies of the *recA* gene). MMS is a DNA-damaging agent that is used to determine the presence of functional *recA*, that is, bacterial cells without *recA* functional involvement in the SOS response (Miller and Kokjohn, 1990) will not grow on MMS. Colonies were only observed to grow from *Pst*I generated pSK- libraries after overnight incubation, and these colonies were shown to contain identical genomic inserts (data not shown). Furthermore, one of the cosmids (p103RecA-1) containing the *recA* gene had the ability to complement *recA*-defective *E. coli* strains, thus providing them the ability to grow on LBA supplemented with MMS and lending support to p103RecA-1 containing a functional copy of the NZ103 *recA* gene.

p103RecA-1 is currently undergoing subcloning in order to determine the minimal sized fragment able to restore RecA activity to *recA* deficient *E. coli* strains. The minimal gene fragment required to restore *recA* activity will be nucleotide sequenced by designing primers that provide contiguous overlapping sequence that will be deposited to GenBank. Once the gene is characterised, sequence information will be used to generate a restriction profile to determine potential cloning sites of an antibiotic cassette to create an insertional mutation with the NZ103 *recA*. The antibiotic cassette (such as Omega-spectinomycin (Ω-Sp) cassette) will be amplified using primers incorporating the selected flanking enzyme sites (identified by NZ103-*recA* nucleotide sequencing) and cloned into the NZ103 *recA* gene. Such a NZ103 mutated *recA* gene fragment will then be used to shotgun clone into *Pseudomonas* specific vectors (such as pME6001 (Heeb, *et al.*, 2000)) to enable future introduction of the mutated *recA* gene into NZ103 strains. Once the pME6001 derivative containing the mutated *recA* gene is introduced into NZ103, the occurrence of homologous recombination and replacement of the functional gene for the mutated one should be selected on LBA supplemented with Sp (used to select *recA* gene containing the Ω-Sp cassette) and subsequently, Sp resistant NZ103 colonies should be shown to exhibit a loss of resistance to UV irradiation and MMS exposure. Furthermore, recombination (rather than plasmid retention) should be tested by the loss of the vector marker (e.g. Tc^R in pME6001) and subsequent Southern analysis.

These procedures to confirm the creation of a *recA* deficient strain of NZ103, if unproblematic, should then be applied to mini-Tn*5*km*lac*Z2 NZ103 mutants to create *recA* deficient mutants that will allow analysis of mutated genes using *trans*-complementation. Such a *recA* deficient system will not only be of great value for genetic analyses of OprF in 103F5, and other NZ103 mutants created in this study, but also any future mutagenesis studies of NZ103.

8.5 Horizontal transfer

Horizontal transfer of genetic elements is likely to occur in the mushroom industry given the temperature regime during the composting process up to harvesting of mushrooms. Compost is made by encouraging diverse microorganisms to colonise in the initial stages to provide an environment where bacteria and fungi produce desired secondary metabolites for *A. bisporus* growth. This heavily inoculated compost is then subjected to peak heat (Chapter 1.1.3) that reaches temperatures of 60-65°C for 12hrs and then 50-55°C for 48 hrs. During this time, mesophilic bacteria and fungi are unlikely to survive and therefore, will die and there cellular contents (including DNA) will become part of the composts microelements. Subsequent colonisation, such as by BCOs described in this study, will be in an environment surrounded by DNA. Temperatures within the compost may reach ca. 40°C, which coincidently, is approximately the same temperature used to induce bacterial conjugation and to inactivate host restriction systems *in vitro*, therefore it is likely that the uptake and integration of some of this foreign DNA may occur. To test this hypothesis in the first instance, temperature profiles should be obtained from within compost and casing during the mushroom cultivation processes. BCOs from this study could be mixed with a selected plasmid DNA (such as pME6031) and subjected to the determined temperature regime. Screening for uptake of such a plasmid could be screened by antibiotic resistance (Tc). Further to this, BCOs could be mixed with a naked piece of DNA such as the LDP isolated from NZI7, and uptake could be screened by PCR amplification and Southern hybridisation. Furthermore, conjugational events could be investigated between BCOs and pseudomonads known to contain conjugative plasmids with suitable markers, for example (Kozdroj, 1997). Investigation of such horizontal transfer would prove interesting in that it might account for the diversity of BCOs able to cause blotch discolourations in this study, and furthermore, it may give an explanation as to why NZI7 was closely related to *P. syringae* (based on 16S rRNA similarity), but had a LDP that had highest similarity to *P. tolaasii* tolaasin.

8.6 Type III secretion processes

Following submission of this thesis, it was identified that a possible scenario that could account for the host response of *A. bisporus* is that of Type III secretion processes (TTSS). TTSS involves the movement of effector molecules between bacteria and its host by an elaborate delivery mechanism. These effector molecules can elicit a hypersensitive response (HR) from the host; that in essence is the identification of foreign proteins which up regulateds a defense response to the bacteria. Given the conclusion of this thesis that the likely induction of tissue discoloration in *A. bisporus* is a host response system to colonizing bacteria, it is plausible that effector molecules may be produced by BCOs and translocated into the mushroom tissue via TTSS. This avenue of investigation will be followed up by Dr Gail Prestion, Oxford University, UK.

8.7 Concluding thought

In any given exploratory study, like that presented in this thesis, many potentially interesting results emerge and are speculated upon because time did not permit experimental data to be obtained. To avoid the demise of ones research into oblivion, it is hoped that results obtained in this thesis will provide a platform from which continuing research will build upon and provide clarity to preliminary observations. It is hoped that the results presented in this thesis are received as interesting observations and that the discussions put forward on the possible models of bacterial blotch are pursued and tested as hypotheses, all with an aim of contributing to the philosophy of science.

APPENDIX I : GENERAL EXPERIMENTAL PROCEDURES

I(i) BACTERIOLOGICAL METHODS

I(i)a Buffers, media, antibiotics and supplements

The composition and preparation of all media, buffers, solutions, reagents and supplements referred to throughout this study, are as described in Appendices II and III.

Antibiotics

Agar plates and liquid cultures used for the growth of bacterial and fungal strains were supplemented with antibiotics, indicators and other supplements where appropriate. Unless otherwise stated, concentrations used, and the abbreviation referred to in this study are described in Table 8-1.

Table 8-1 Antibiotics used during this study. The abbreviation for respective antibiotics is presented along with the working concentration used (unless stated otherwise).

Supplement	Abbreviation	Concentration
Ampicillin	(Ap)	100 $\mu g.ml^{-1}$
Kanamycin	(Km)	50 $\mu g.ml^{-1}$
Gentamicin	(Gm)	30 $\mu g.ml^{-1}$
Nalidixic acid	(Nal)	30 $\mu g.ml^{-1}$
Rifampicin	(Rif)	50 $\mu g.ml^{-1}$
Cycloheximide	(Cx)	100 $\mu g.ml^{-1}$
Streptomycin	(St)	50 $\mu g.ml^{-1}$
Spectinomycin	(Sp)	100 $\mu g.ml^{-1}$
Tetracycline	(Tc)	15 $\mu g.ml^{-1}$

5-Bromo-4-chloro-3-indolyl-i3-D-galactopyranoside ((X-gal) Melford Laboratories) 45 µl ml^{-1} was used as the chromogenic indicator of β-galactosidase activity on agar plates. Isopropyl-β-D-galactopyranoside ((IPTG); Melford Laboratories) 0.5mM was used to induce the *lacZ* gene. Pseudomonas C-F-C Supplement (Oxoid Ltd) was a commercially available product containing cephalosporin as the selective component for pseudomonad growth, and counter-selection of *E. coli* growth; used in trans-conjugant and transformant selection.

I(i)b General culturing of bacterial strains used during this study

Bacterial isolates used in this study are listed in Appendix IV. Reference strains were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, UK, and pseudomonad isolates from a milk factory environment were obtained from a previous study (Reid, 1997). All pseudomonad and *E. coli* strains were incubated at their optimal growth temperatures of

28°C and 37°C respectively. For day-to-day use, strains were maintained on appropriately supplemented agar plates at 4°C for a maximum of two weeks. For long-term storage, strains were suspended in LB plus 15% glycerol and stored at -80°C. Single colonies obtained from selective plates were used to inoculate LB broth (containing appropriate selection) and subsequently cultured for 16 hours. Unless otherwise stated, bacterial cells were harvested by centrifugation at 7000 rpm for 3 minutes. Viable cell counts were carried out by spotting replicate 10 ml aliquots of serially diluted bacterial cultures onto LB agar plates.

I(i)c Low resolution growth analysis

Low-resolution growth analyses were carried out to compare the growth rates over time of selected genetically modified bacteria to the wild type. Overnight cultures were grown in the respectively supplemented LB media and the following day all cultures were diluted 1:1 with LB and OD_{600} readings were performed on a spectrophotometer (Thermo Spectronic, Heliosα, Cambridge UK). OD_{600} readings were used calculate the necessary dilution factor to give each strain to an OD_{600} of 0.500 and 100μl of this was used to inoculate a 100ml of LB in a 250ml flask. Flasks were incubated (28°C, 200rpm) and at pre-determined time intervals, 1ml aliquots were aseptically removed and used to obtain OD_{600} readings.

I(i)d Motility assays

Motility agar was made up as described in Appendix II and because of the low agar content, plates were left to set overnight. To ensure plasmid maintenance where necessary, motility agar was supplemented with the appropriate antibiotics. The motility of pseudomonads were assessed by touching the surface of the agar with a toothpick containing inoculum derived from a single colony and incubating the plate upright at 28°C for 24hrs. Motile pseudomonads were indicated by the presence of a swarm pattern originating from the point of inoculation whereas non-motile bacteria grew but did do not move away from the point of inoculation (Figure 5-4).

I(i)e Transmission electron microscopy (TEM)

Bacterial strains were scraped from an overnight LB plate supplemented with the appropriate antibiotics and resuspended in 8 drops ddH_2O. To these suspensions, one drop of a 2% aqueous solution of phosphotungstic acid were added and mixed. A drop of this suspension was placed on a carbon-coated TEM grid and excess aqueous solution drained off by capillary action. Negatively stained bacteria were examined with a JEOL JEM-1200EX Electron microscope.

I(ii) ASSAYS FOR BACTERIAL/MUSHROOM INTERACTIONS

I(ii)a Isolation of pseudomonads from mushroom farms

Sampling from selected mushroom farms was carried out by excising a section of tissue from selected mushroom caps exhibiting blotch discolourations and was placed into a McCartney tube containing sterile KB medium (10 ml). One gram of compost or casing material was placed into a McCartney tube containing sterile KB medium (10 ml). Water samples from water reservoirs and frequently used taps on mushroom farms were collected in sterile McCartney bottles. Samples were maintained on ice upon collection and during transport to the laboratory, a period not exceeding 2 hr. Samples were incubated for 24 hr at 28°C before an aliquot was applied to Gould's agar medium (Gould, *et al.*, 1985). Selected individual bacterial colonies were purified by passage onto fresh Gould's medium and stored as above.

I(iii) DNA MANIPULATION

I(iii)a General

DNA concentration was determined by either: (i) electrophoresis through agarose gels, followed by subsequent staining of DNA with ethidium bromide (EtBr) and viewing on a UV transilluminator in comparison to DNA standards (e.g. 1KB Plus ladder, or λHindIII); or (ii) by measuring the ratio of absorbance at 260nm relative to the absorbance at 280nm using an LKB Ultraspec Plus spectrophotometer with deuterium lamp. All isopropanol, 70% ethanol, and 100% ethanol steps referred to for the purpose of precipitation of DNA out of solution, were stored at -20°C prior to use. DNA obtained was maintained at either 4°C or at -20°C.

I(iii)b Preparation of genomic DNA

1. Promega Wizard Kit

The Wizard® Genomic DNA Purification Kit (Promega) was used generally for the extraction of genomic DNA from *Pseudomonas* and *E. coli* strains as per manufacturers instructions.

2. Genomic extraction from Pseudomonas

The cells from 1.5ml of an overnight culture were harvested in an eppendorf tube by centrifugation. The supernatant was removed by aspiration, the cell pellet was resuspended in 300µl of TES and the cells were again collected by centrifugation. After resuspending the cell pellet in 100µl of 25% sucrose in TE, fresh lysozyme solution was added (to a concentration of 1 $mg.ml^{-1}$). After a 15 min incubation at 37°C, 20µl of 10% SDS, 5µl of RNaseA ($10mg.ml^{-1}$), and 200µl of ddH_2O were added. The solution was mixed by gentle rotation and inversion, and then incubated at 37°C until lysis of all cells had been achieved (typically this was visualised by the clearing of the solution). Once clear, 5µl of proteinase K ($10mg.ml^{-1}$) was added and the solution was incubated for a

further hour at 37°C. The solution was transferred to a 10ml siliconised corex tube, and 350µl of TE was added. To this, 700µl of phenol and 700µl of chloroform:isoamyl alcohol (24:1) were added and the three components were mixed by repeated inversion until a single emulsion formed. Following centrifugation (10 min, 13000 rpm, 4°C), the clear top (aqueous) phase was removed using a Pasteur pipette, carefully avoiding the white precipitate at the interface. Approximately 500µl of the clear aqueous layer was transferred into each of two eppendorf tubes. The phenol:chloroform:isoamyl alcohol extractions were repeated until no precipitate was seen at the interface. A single extraction with 2x volumes of chloroform:isoamyl alcohol followed to remove any remaining phenol residues. The DNA was then precipitated from the aqueous phase by adding 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% ethanol. The DNA was carefully wound onto the end of a heat sealed Pasteur pipette and allowed to briefly air dry before dissolving it in 50µl of TE.

I(iii)c Preparation of plasmid DNA

All of the following plasmid extraction methodologies are based on the alkaline lysis method (Birnboim and Dolly, 1979) that works on the following principle. The cells are lysed in the presence of NaOH and SDS with the addition of RNAse. The SDS denatures cellular proteins and the alkaline conditions ensure that both plasmid and chromosomal DNA becomes denatured. The lysate is neutralized and adjusted to high salt binding conditions. The chromosomal and plasmid DNA are separated as the chromosomal DNA precipitates together with the cellular debris under high salt binding conditions leaving the plasmid DNA in the lysate. During neutralisation and high salt adjusting step, the smaller plasmid DNA re-anneals correctly and stays in solution. The chromosomal DNA and cellular debris are removed by centrifugation while plasmid DNA is adsorbed onto a column of positively charged silica and subsequently eluted with 10mm Tris-HCl buffer (pH 8.0) and stored at -20°C.

Qiagen Method.

This method was used for high quality plasmid preparation for cloning and nucleotide sequencing of vector inserts. Plasmid DNA was isolated from *E. coli* strains using the QIAprep® Spin Miniprep Kit (Qiagen Laboratories) according to manufacturers instructions. The Qiagen kit protocols are based on the alkaline lysis method (Birnboim and Dolly, 1979) described above.

"mini-prep" plasmid extraction (Birnboim and Dolly, 1979, Sambrook, et al., 1989)

This method was used for screening multiple transformants and preliminary RFLP analysis. Cells from 1.0ml to 100ml of an overnight culture grown in LB were harvested by centrifugation, and the pellet resuspended in 0.1x volumes of solution I (Appendix II) by vortexing. Solution II was added to 0.2x the original culture volume and mixed by gentle rolling until the solution became clear and viscous, indicating complete cell lysis. Solution III was added to 0.15x the original culture volume and mixed by inversion until a white precipitate in a clear solution had formed. The solution was kept on ice or at -20 °C for 10min. The solution was then separated by centrifugation at 13,000 rpm for 10

min. The supernatant was decanted into a new centrifuge tube, and 1x volume cold 100% ethanol added and mixed well. For medium to high copy number plasmids the precipitated DNA was immediately collected by centrifugation (13,000rpm, 10min), whereas for low copy number plasmids the solution was kept on ice for 5-10min prior to centrifugation. The supernatant was removed by aspiration and the pellet washed with 1ml of room temperature 70% ethanol. After centrifugation (13000rpm, 5min), the 70% ethanol was removed by aspiration and the pellet air dried briefly. The DNA pellet was redissolved in the appropriate volume of sterile ddH_20. The volume of ddH_20 used depended on the volume of the original culture as well as the copy number of the vector, but in most cases was between 20µl and 50µl.

I(iii)d Restriction endonuclease digestion of DNA

Restriction digestions were routinely performed in 20-50µl volumes and were incubated at the appropriate temperature using 4 units of enzyme per 1µg of DNA. Multiple restriction digests were carried out in the most appropriate reaction buffer for both enzymes as determined by the respective manufacturers. Alternatively, the DNA was first digested with the enzyme requiring a low salt buffer in low salt conditions and when digestion was complete, the high salt buffer and the second enzyme were added and the digestion reaction continued for a further two hours. In some cases, it was necessary to phenol extract the DNA after the first digestion to remove enzyme and salt prior to digestion of the DNA with the second enzyme. The appropriate control digestions were performed in all cases.

I(iii)e Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to separate DNA fragments obtained from restriction digests. All agarose gels run in this study had a minimum of one lane containing a standard DNA marker (either a 1KB Plus ladder, or 100bp Ladder (Roche)) to act as reference for both DNA concentration, and DNA fragment size. Gels contained DNA-grade agarose (Bio-Rad) and buffered with 0.5x TBE (Appendix III) to give agarose concentrations of 0.4-1.2 % depending on the size of the DNA fragments to be separated. DNA was visualised using UV light, by either a) adding ethidium bromide (Sigma) $10\mu g.ml^{-1}$ to the gels prior to pouring; or b) staining the gels in a solution of ethidium bromide ($0.5mg.ml^{-1}$ in 0.5x TBE) for 10-20 minutes, and when required, de-staining was achieved in a solution of 0.5x TBE for 10-20 minutes. Stained gels were viewed on a Sigma T2210 UV transilluminator (302nm) or using an Ultralum KS3000 gel Visualisation-Documentation and Analysis system, allowing digital images to be saved.

I(iii)f Extraction and purification of DNA from agarose gels

Desired DNA fragments were isolated from agarose gels following endonuclease digestion, and purified using a QIAquick® Gel Extraction Kit (Qiagen Laboratories) according to manufacturer's instructions. The agarose was solubilized by the addition of a chaotropic salt that disrupts hydrogen

bonding between sugars in the agarose. The DNA was adsorbed to silica-gel particles in the presence of high salt and subsequently eluted in TE buffer. All non-nucleic acid impurities including agarose, proteins, salts and ethidium bromide were removed during washing ste*P*.

I(iv) POLYMERASE CHAIN REACTIONS (PCR)

I(iv)a Oligonucleotides and primer design

All oligonucleotides and PCR conditions used in this study are described in Appendix VI. Oligonucleotide primers were synthesized (Genset Oligonucleotide Facility, Singapore) were maintained at –20°C in ddH_2O at a stock concentration of 50µM. Unless stated otherwise, oligonucleotide primers were designed using Primer Express™ Software (Perkin Elmer) to be vector-specific or gene-specific primers. For sequencing in from the multiple cloning site (MCS) of pSK+ and pKS+ the T3 and T7 universal primers (Promega) were used (Appendix VI).

I(iv)b Standard PCR conditions

The template for PCR amplification was double-stranded DNA of a chromosomal or plasmid origin. All PCR amplifications were carried out in a Perkin Elmer 9700 thermocycler. Unless stated otherwise, a standard PCR reaction mixture (25 ml total) consisted of 1x buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl2, 0.01% gelatin, and 0.1% Triton X-100), deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP) at a final concentration of 200 mM, 0.625 U of Taq DNA polymerase (Roche Molecular Biochemicals), oligonucleotide primers (Appendix VI) at a final concentration of 2 mM, and 100 ng of template DNA. Thermocycling parameters were carried out according to the oligonucleotides used and the size of amplicon required (Appendix VI). Prior to cycling, samples were heated at 94°C for 5 min and the extension step was increased to 5 min, 72°C as part of the terminal cycle. Primers and dNTPs were removed from PCR products using the High Pure® PCR Product Purification Kit (Roche Molecular Biochemicals).

I(iv)c 16S rRNA amplification and sequencing

Primers U16A and U16B (Appendix VI) were used to amplify the nearly complete 16S rRNA gene (approx 1480 bp). Direct nucleotide sequencing of this gene was achieved using primers U16A, F357, F945, U16B, R1087 and R518 (Appendix VI) in combination with the Big Dye Terminator Kit and an ABI Prism 3TIXLCPE (PE Biosystems). All 16S rRNA gene sequences analyzed in this study were confirmed by determining contiguous overlapping sequences of PCR-DNA. The 16S rRNA gene sequences determined in this study have been deposited with GenBank under accession numbers listed in tables within the results of corresponding chapters.

I(iv)d Repetitive extragenic polymorphic (REP) PCR

The primers (REP1R-I and REP2-1 (Appendix VI)) and protocols used for REP-PCR were those described by de Bruijn (De Bruijn, 1992).

I(v) DNA SEQUENCING PCR REACTIONS

I(v)a Preparation of DNA for sequencing

DNA was cloned according to standard molecular biology techniques (Sambrook, *et al.*, 1989). Commercially available DNA preparation kits were used exclusively to provide high quality DNA for sequencing and kits used were:

PCR products, digested plasmids (between 100bp – 40KB)	High Pure PCR Product Purification Kit (Roche)
Extraction of DNA from agarose gels	QIAquick® Gel Extraction Kit (Qiagen)
Plasmid DNA extracted from *E. coli* (DH5α, DH10B)	QIAprep® Spin Miniprep Kit (Qiagen)
Genomic DNA extraction	Promega Wizard Kit (Promega)

Automated sequencing using Large Dye Terminator Kit (ABI)

The template for sequencing was either clean double-stranded plasmid DNA or clean gel extracted PCR amplicons and the primers were vector-specific or gene-specific primers. PCR sequencing reactions were carried out to prepare and incorporate labeled terminator nucleotides using the Big Dye 3.0 (Perkin Elmer) as advised by the respective sequencing facilities. Following the PCR sequencing reactions, the DNA was precipitated according to the following protocol to remove enzymes, unincorporated dNTPs and "large dye" terminators (ABI). DNA was removed from the 0.2ml PCR tube and put into a 1.5 ml microfuge tube. 80 µl of ddH_2O water added to bring the volume to a total of 100µl. A 0. 1 volume of NaOAc (pH 5.2) and 2.5 volumes of 95% cold ethanol were added and the DNA was left to precipitate at room temperature for a maximum of 10 minutes. This was followed by centrifugation (15 min., 13000 rpm) and a 70% ethanol wash. The DNA pellet was air dried. At this stage, samples were sent to a sequencing facility and automated sequencing was performed on an Automated ABI 310 (Waikato sequence facility, Waikato University, New Zealand).

I(vi) NUCLEOTIDE SEQUENCE ANALYSIS

I(vi)a Computer analysis of DNA sequences

Nucleotide and derived protein sequences were analyzed using the Sequencher™ 3.0 sequence analysis software package (Gene Codes Corporation). DNA and amino acid sequence homology searches were performed at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/blast) with a BLAST network server and the non-redundant DNA and protein sequence databases.

I(vi)b Phylogenetic analysis of 16S rRNA gene nucleotide sequences

The 16S rRNA gene nucleotide sequences obtained in this study were aligned with selected 16S rRNA sequences obtained from GenBank using the nucleotide alignment software Clustal W (Thompson, *et al.*, 1994). Phylogenetic trees were constructed with neighbor joining (Saitou and Nei, 1987) and evolutionary distances calculated according to Jukes and Cantor (Jukes and Cantor, 1969) using the software package Treecon for Windows version 1.3b (Van de Peer and De Wachter, 1994). Bootstrap analysis (Felsenstein, 1985) was carried out using 500 replicates. *Acinetobacter calcoaceticus* ATCC 23055 was included for single sequence (forced) outgroup rooting of the tree.

I(vii) CLONING STRATEGIES

I(vii)a Preparation of DNA for cloning

DNA was cloned according to standard molecular biology techniques (Sambrook, *et al.*, 1989). Commercially available DNA preparation kits were used exclusively to provide high quality DNA to enhance cloning out comes (as described for sequencing DNA preparation).

I(vii)b Dephosphorylation of DNA

Dephosphorylation using calf intestinal phosphatase (CIP) removed the terminal 5' phosphates of digested DNA with the aim of prevented self-ligation of the vector. The DNA to be CIP treated was precipitated, redissolved in 44µl ddH_2O, and 5µl of 10x dephosphorylation buffer (Böehringer Mannheim) and 1 unit CIP (Böehringer Mannheim) were added. The reaction was incubated at 37°C for 30min before being stopped by the addition of EDTA to 5mM and incubation at 75°C for 10min. The volume was made up to 200µl with ddH_2O, and the DNA extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by the addition of 0.125x volumes potassium acetate (pH 8.0) and 2.5x volumes 100% ethanol, and incubation at -80°C for 10-15min. The DNA was collected by centrifugation, and the pellet air dried briefly and redissolved in 10µl ddH_2O.

I(vii)c DNA ligation

For the ligation of compatible cohesive termini, insert and vector DNA were ligated using T4 DNA Ligase (Gibco) at a molar ratio of 3:1 (insert:vector). Ligation reactions were made up to a 20µl volume with sterile ddH_20 and ligation buffer (supplied by the manufacturer). Ligations were performed overnight at room temperature. Ligation reactions of blunt-ended DNA were performed as for cohesive termini with exception of the insert and vector DNA were ligated at a molar ratio of 1: 1. Following respective ligation, DNA was treated as required for either electro transformation or conjugation described within this Appendix.

I(vii)d Subcloning

Subcloning was used within Chapter 6.3 to subclone NZ103 DNA fragments containing mini-Tn*5*km*lacZ*2 from pBluescript SK- (pSK-) to pBR322 when required. pSK- was digested with a selected restriction enzyme site that excised the desired DNA fragment and the same enzyme was used on a unique cloning site in pBR322. The completion of the digest was verified by agarose gel electrophoresis. Enzymes from the restriction digest were removed by a phenol:chloroform extraction followed by an ethanol precipitation step, and the DNA was re-suspended in TE buffer. The total DNA was re-ligated to produce a range of subclones within pBR322. This pool of subclones was used to transform *E. coli* DH10B or *E. coli* DH5α and transformants were selected on LBA supplemented with Tc (to select for pBR322 and counterselect pSK-) and Km (to select for mini-Tn*5*km*lacZ*2).

I(viii) ELECTROPORATION

I(viii)a Preparing electro-competent cells

E. coli strains DH10B, S 17-λpir, or DH5α λpir were used to make competent cells using methods based on the desired *E. coli* strain was used to inoculate 5 ml of LB broth and incubated overnight at 37°C with vigorous shaking (200 rpm). The following day, a 50-100ml culture was initiated by diluting an overnight culture 1:100 into fresh LB. These were grown to exponential phase (OD600 of 0.5 to 0.8 – ca. 5 x 10^7cfu.ml^{-1}) and chilled on ice for 20 minutes. The cells were gently pelleted by centrifugation (10 min, 4500 rpm, 4°C), resuspended in the original volume of cold ddH_2O, pelleted by centrifugation (10min, 5000 rpm, 4°C) and resuspended in half the original volume of cold ddH_2O. Finally, the cells were pelleted by centrifugation (10min, 5500 rpm, 4°C), resuspended in 250-500μl ddH_2O and divided into 40μl aliquots for immediate use. Competent cells for storage were resuspended in 1-2 ml of 10% glycerol, collected by centrifugation (10 min, 5500 rpm, 4°C), and resuspended in 250-500μl of 10% glycerol before dividing into aliquots and storing at -80°C. *E. coli* cells which have been rendered competent for transformation were stored at –80°C for periods of up to three months.

I(viii)b Preparation of DNA for electroporation

Treatment of plasmid DNA to remove salts and other contaminating products was carried out to prevent inefficient transformation events. This was achieved by using one of the two following methods:

Concentration by ethanol precipitation

Desired DNA for electroporation was made to a volume of 100μl with ddH_2O, 1 ml of cold 100% EtOH was then added along with 5μl of 3M NaAc. This was mixed and placed at -80°C for 10min. Following centrifugation (13,000rpm, 10min, 4°C), EtOH was removed by aspiration, and the pellet was washed with 1 ml of 70% EtOH. Following centrifugation (13,000rpm, 10min, 4°C), the 70%

EtOH was again removed by aspiration. The pellet was dried in a vacuum manifold and redissolved in 10-30μl of ddH_2O.

Salts were removed by Filter dialysis

Filter dialysis of DNA for electroporation involved using a sterile Petri dish containing ca. 20 ml of ddH_2O and a 0.025μm-nitrocellulose filter (Millipore Corporation) placed onto the water surface. No more than 15μl of O/N ligation, or enzyme digested DNA, was placed gently on the filter paper and left for no less than 30 min at RT. The membrane pores are small enough to let salts pass via osmosis, however the pore size does not permit DNA to pass. DNA was removed from the filter paper and the desired amount was used for electroporation.

I(viii)c Electrotransformation

Electroporation (Dower, *et al.*, 1988, Zabarovsky and Winberg, 1990) was carried out using a Bio-Rad Gene PulserÔ and pulse controller with capacitance set at 25mF and resistance at 200 W, and a voltage of 1.8 kV for cuvettes with a 0.1cm gap or 2.5 kV for cuvettes with a 0.2cm gap. When fresh electro-competent cells were used, they were kept on ice, while stored cells were thawed at room temperature before being placed on ice. The electroporation slide chamber and sterile cuvettes (Bio-Rad Gene Pulser electroporation cuvette (Bio-Rad)) were also chilled prior to use. Approximately 100ng of DNA in ddH_20 was added to the cells, mixed well, and kept on ice for one minute before transferring the cells to a cuvette. The mixture of competent cells and DNA was pipetted gently into the cuvette (0.1 cm electrode gap) and the cuvette tapped briefly to disperse cells in the bottom. The cuvette was placed quickly into the cuvette holder and cells were electroporated at the above settings, and immediately following charge release 1 ml of pre-warmed (37°C for *E. coli* and 28°C for pseudomonads) SOC or LB was added to the cuvette. Cells were then transferred to a sterile eppendorf tube and elaborated at the appropriate temperature for at least one hour with gentle agitation. Aliquots were spread on selective media and incubated at the appropriate temperature overnight.

I(ix) $CaCL_2$ TRANSFORMATION BY HEAT SHOCK

I(ix)a Preparation of competent cells for transformation by heat shock

A single colony of *E. coli* strain was used to inoculate 5 ml of LB broth and incubated overnight at 37°C with vigorous shaking (200 rpm). 1 ml of the overnight culture was used to inoculate 100 ml of LB broth in a 250 ml conical flask with the addition of 10mM $MgSO_4$ and 10mM $MgC1_2$, and the incubation was continued until a cell-density of ca. 0.5 (OD 600) had been reached (~5 x 10^7 cfu.ml^{-1}). Cells were chilled on ice for 15 minutes and kept as cold as possible for the remainder of the procedure. Cells were pelleted by centrifugation at 4°C, 7000 rpm., 15 min. The supernatant was removed and the pelleted cells were re-suspended in a volume of ice cold RF1 (Appendix III) that was

1/3 of the original volume collected. The cells were incubated on ice for 30 mins. The cells were pelleted as above and all traces of supernatant were removed. The cells were re-suspended in RF2 (Appendix III) buffer that was 1/20 of the original volume, and incubated on ice for 15 min. The cells were distributed in 100μl aliquots and flash frozen in liquid nitrogen before storage at -80°C.

I(ix)b Transformation of competent cells by heat shock

100µl of competent cells were thawed on ice and approximately 10ng of plasmid DNA (in a volume of 20μ1) was added to the competent cells. DNA and competent cells were mixed gently and left on ice for 1 hour. The cells were heat shocked by placing in a water bath at 42°C for 3 minutes and returned immediately onto ice for a further 5 minutes. Cells were added to 1 ml of pre-warmed SOC (Appendix III) and incubated at 37°C with gentle agitation to allow recovery of the cells and expression of the antibiotic resistance. The transformation mixture was spread onto LB selection plates containing the appropriate selective agent(s).

I(x) SCREENING TRANSFORMANTS AND TRANSCONJUGANTS

To validate authenticity of the incorporation of plasmid and/or genes introduced into the desired bacterium, the following screening strategies were employed.

I(x)a Antibiotic selection

Addition of an antibiotic to a growth substrate enabled selection of organisms with the desired acquisition of a plasmid/gene containing nucleotide sequence that encodes antibiotic resistance. To confirm the loss of antibiotic resistant plasmid vector, or the loss of a gene encoding antibiotic resistance, colonies were replica-plated on media containing absence and presence of antibiotics.

I(x)b Blue/white screening of recombinant clones

Blue/white screening was used to determine cloning events within vectors containing multicloning sites in the β-galactosidase gene. The chromogenic substance, X-gal, and the inducer IPTG were added in addition to the antibiotic (if required) in the selection medium. Recombinant DNA molecules arise by insertional inactivation of the *lac*Z gene, and recombinant clones can be distinguished by their inability to produce β-galactosidase therefore giving rise to white colonies, whereas unaltered *lac*Z activity gives blue colonies.

I(xi) MLEE

The MLEE methods used were derived from previous studies (Selander, *et al.*, 1986, Haubold and Rainey, 1996) for enzymes glucose-6-phosphate isomerase (GPI); glucose-6-phosphate dehydrogenase (G6PDH); malate dehydrogenase NADP (ME); and 6-phosphogluconate dehydrogenase (6PGDH).

Protein extraction

Protein extraction from whole cells using the mini bead beater was modified from Herrick et al. Respective cells were cultured overnight in 10ml LB broth, and the following day were harvested and the cell pellet was resuspended in 1ml Extraction buffer (Appendix III). 1ml of Extraction buffer containing cell suspension was placed into a sterile 2ml polyproplene screw cap tube containing 2g of autoclaved 0.1mm Zirconium silicate beads (BioSpec Products). Each tube was shaken in a Bead mill homogenizer (BioSpec Mini-Beadbeater, Biospec Products) for 1min at 3800 strokes min-1 in bead mill homogenizer, placed on ice (5 min) and repeat. The supernatant was collected by briefly (<5sec) centrifuge tubes to pellet beads and the supernatant was transferred to a sterile 1.5ml eppendorf tube. 500ml of Extraction buffer was added to the remaining beads and vortexed briefly, centrifuged and the aqueous crude extract was pooled in the same 1.5ml eppendorf tube. A final centrifuge (3min, 8000 rpm) was performed to remove any remaining beads and the aqueous layer was transferred to a new 1.5ml eppendorf tube and store at -80°C until required for MLEE.

Non-Denaturing Electrophoresis on Cellulose Acetate

A Titan III cellulose acetate gel apparatus (Helena Laboratories) was used in this study. Initially Titan III cellulose acetate plates were cut into quarters (76mm by 76mm) and soaked in a beaker containing 400ml of TG buffer (see Appendix III). Care was required when immersing plates into the TG buffer to prevent blistering of the cellulose. The cooling tray was cooled to 4°C and placed into the dry well in the centre of the electrophoresis tank. Both sides of the electrophoresis tank were filled with 200ml of TG buffer. The cellulose acetate plates were removed from the TG buffer and blotted dry with Whatman 3mm filter paper. 10ml of each sample was loaded into each sample well in the sample loading plate (maximum of 12 samples). This sample was transferred to the cellulose acetate plate using the Super Z-12 applicator apparatus (Helena Laboratories) as per the manufactures instructions. The plate was placed acetate side down on the centre of the cooling tray in the electrophoresis tank. Each end of the plate was covered by a filter paper wick soaked briefly in TG buffer. The wicks had one edge immersed in the tank buffer and the other covering approximately 5mm of the cellulose acetate plate along one entire edge. Electrophoresis was carried out at room temperature at 200 V for approximately 15min.

Enzyme Staining for Cellulose Acetate

Cellulose acetate plates were removed from the cooling tray in the electrophoresis tank and place into a large plastic weighing boat. The plate was covered by 5ml of the appropriate staining solution (see Appendix III) and incubated at 37°C until clear, distinct band staining is apparent (approximately 10min). The enzymes GPI, G6PDH, ME, and 6PGDH were analysed. Scoring of electromorphs was performed as previously described (Selander, *et al.*, 1986), and cluster analysis was carried out using unweighted pair group algorithm averages with the S-Plus statistical analysis package (version 4.5; MathSoft Inc., Seattle).

I(xii) TRANSPOSON MUTAGENESIS

I(xii)a Generation of insertional mutations using mini-Tn*5*km*lacZ2*

Mini-Tn*5*km*lac*Z2 was available as an insertion in the pUT vector and will be referred to as pUTZ2 (de Lorenzo, *et al.*, 1990). pUTZ2 contains the RP4 origin of transfer so is mobilisable by conjugation when tra functions are supplied in trans and *E. coli* S17-1λpir can provide such functions as it has an RP4 derivative plasmid integrated into its chromosome (Simon, *et al.*, 1983).

E. coli S17-1λpir containing pUTZ2 was conjugated with NZ103 (Chapter 5.3). Aliquots of 10mL were removed from washed donor and recipient suspensions for control purposes. Donor and recipient cells were mixed together thoroughly and 3 aliquots of 60mL spotted separately onto pre-warmed LB plates. All plates were incubated at 28°C for 1 hour before being recovered off the plate and plated on LB plates supplemented with Km and CFC (selection for pseudomonads). pUT is a suicide vector which has its oriV derived from the R6K plasmid and as a result of this, it cannot replicate without the p protein being supplied in trans. For this reason colonies present on selective plates were deemed to be transformants of NZ103 that had obtained Km resistance due to the transposition of mini-Tn*5*km*lac*Z2 into its chromosome.

I(xii)b Screen for PPDs of BCOs and NZ103 mutants

Individual transformants were toothpick transferred from selective plates onto both a reference plate containing Km in the grid pattern correlating to the wells of half of a microtitre dish (Figure 5-2). No more than 100 transformants were taken from each individual plate. Assays were set up as described in Chapter 4, to test PPDs using the multi-pronged replica plater device (Figure 5-2). This device allowed the simultaneous transfer of 48 colonies from reference plates to either supplemented agar or abiotic biofilm assay micro titre plates. In between each transfer the replica-plater was sterilised with flaming alcohol and cooled down by emersion in sterile distilled water

I(xii)c Abiotic biofilm assay and staining proceedure

This method was derived from a previous study (O'Toole and Kolter, 1998b). Microtitre wells containing 98μL of L10 media (Appendix II) were inoculated with 2μL of overnight culture (1:50 dilution), covered with an inverted microtitre plate and incubated at 28°C. After 16 hours of incubation, 10μL of Gram's Crystal Violet solution (BDH Laboratory Supplies) was added to each well and allowed to stain for 20min. At such time, wells were rinsed vigorously with water until all excess crystal violet had been removed. Excess water was taped out on to a paper towel and the wells air-dried. Biofilm formation was indicated by the presence of a ring of stained bacteria adhered to the well at the oxygen/media interface (Figure 4-3). Visualisation of biofilm 'rings' within individual wells was achieved by carefully cutting up microtitre dishes and viewing on a white-light

transilluminator. Every microtitre dish had at least two positive controls of the wildtype NZ103 and two negative controls of uninoculated bacteria to enable comparisons.

I(xii)d Screening and storage of NZ103 mutants

Any NZ103 mutants that showed defects in phenotypic expression of one of many PPDs were transferred to a new reference plate and retested in a manner identical to that of the first screen. Those mutants that maintained their phenotypes were streaked on selective plates to obtain single colonies. Isolates exhibiting consistent mutant phenotypes for at least 3 replicate assays were cryogenically stored at -80°C and referenced in relation to the grid position assigned. As a final check, the culture used to store the strain was revived from -80°C and tested for its mutant properties.

APPENDIX II : MEDIA

All chemicals used in this study were of reagent grade. Where specific media and reagents are used the method is given. All stock solutions were sterilized by autoclaving (121°C, 22 min) unless otherwise specified.

Luria Bertani media (LB) (Sambrook, *et al.*, 1989)

Bacto tryptone	1% w/v
Yeast extract	0.5% w/v
NaCl	0.5% w/v
In ddH_2O; autoclaved	

L10 media LB media diluted 1:10 with ddH_2O, autoclaved

LB agar (LBA) LB media with 1.8% w/v agar

Kings B agar (King, *et al.*, 1954)

Bacto tryptone	2% w/v
K_2HPO_4 anhydrous	0.15% w/v
$MgSO_4.7H_2O$	0.15% w/v
Glycerol	1.5% w/v
Agar	2% w/v
In ddH_2O; autoclaved	

PAF media (Difco)

Bacto Tryptone	10g
Bacto Proteose Peptone No.3	10g
Di-potassium phosphate (K_2HPO_4)	1.5g
Magnesium sulfate ($MgSO_4.7H_2O$)	1.5g
Bacto Agar	15g
In 1L ddH_20; pH 7.0; autoclaved	

SOC media (Sambrook, *et al.*, 1989)

Bacto tryptone	2% w/v
Yeast extract	0.5% w/v
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	0.36% w/v
In ddH_2O; autoclaved	

5x M9 salts media

$Na_2HPO_4.7H_20$	6.4% w/v
KH_2PO_4	1.5% w/v
NaCl	0.25% w/v
NH_4Cl	0.5% w/v
In ddH_2O; autoclaved	

M9 / glucose (1%) agar

SOLUTION A:

5x M9 salts	200ml
ddH_2O to 500ml; autoclaved	

SOLUTION B:

Agar	1.5 % w/v

ddH_2O to 500ml; autoclaved.

Solutions A and B were cooled, mixed together along with 20ml of 20% glucose solution (filter sterilized), and then poured

Pigment Production Media (P.P.M.) (Levich and Stadtman, 1964)

Peptone	2 % w/v
Glycerol	1 % w/v
NaCl	0.5 % w/v
KNO_3	0.1 % w/v

In ddH_2O, pH adjusted to 7.2; autoclaved

Pseudomonas Minimal Media (PPM) (Kirner, *et al.*, 1996)

SOLUTION A

$K_2HPO_4.3H_2O$	35 mM
KH_2PO_4	22 mM
$(NH_4)_2SO_4$	8 mM

ddH_2O to 500ml; autoclaved

SOLUTION B

Agar	1.5 % w/v

ddH_2O to 500ml; autoclaved

Solutions A and B were cooled, mixed together along with 25 mM sodium succinate (filter sterilised) and 1.2 mM $MgSO_4$ and then poured

Milk Agar (Protease) Plates

SOLUTION A

non-fat milk powder	20g
ddH_2O	200ml

Autoclaved (10min only), store at 37°C

SOLUTION B

LB Agar ingredients for 1000ml

ddH_2O	800ml

Autoclave

Once Solution B at 55°C, mix with solution A and pour

Sierra (Lipase) Medium (Atlas, 1995)

SOLUTION A:

Agar	15g
Peptone	10g
NaCl	5g
$CaCl_2.H_20$	0.1g
ddH_2O	990ml

Autoclave

SOLUTION B:

Tween™ 80	10ml

Autoclave separately (121°C, 15min)

Once Solutions at 55°C, mix and pour

Motility agar (R. Monds, Pers Comm)

NaCl	0.45%
Agar (for a 0.3% agar plate)	0.3%w/v
Agar (for a 0.45% agar plate) etc	0.45%w/v

In L10 media; autoclaved.

***Chitinase Agar* (Draborg, *et al.*, 1996)**

Crab-shell chitin stained with remazol brilliant violet (Sigma, USA) was dissolved to a final concentration of 0.83% (w/v) in hydrochloric acid (37%) and stirred in a shake flask for 2 h. The chitin was washed with water until pH 3 and sterilized by autoclaving. Agarose plates of various media (e.g. LB or M9 (as above)) were made by adding 0.2% colloidal hydrated chitin. Plates were incubated at 28°C for until halos were visible.

APPENDIX III : BUFFERS AND SOLUTIONS

III(i) COMMON BUFFERS (SAMBROOK, *ET AL.*, 1989)

T10E1 (TE)	Tris-HCl	10 mM
	EDTA	1 mM
	In ddH_2O; pH adjusted to 8.0.	
TES	Tris-HCl	10 mM
	EDTA	1 mM
	NaCl	100 mM
	In ddH_2O; pH adjusted to 8.0.	
TBE	Trizma base	50 mM
	Boric acid	50 mM
	EDTA	1 mM
	In ddH_2O; pH adjusted to 8.0.	
DNA Loading Buffer	Glycerol	30 % w/v
	Bromophenol blue	0.25 % w/v
	Xylene cyanol	0.25 % w/v
	RNaseA	10 $mg.ml^{-1}$

III(ii) SPECIFIC BUFFERS AND SOLUTIONS

Alkaline lysis of DNA		
Solution I. (Stored at 4°C)	Glucose	1 % w/v
	Tris-HCl, pH 8.	025 mM
	EDTA	10 mM
	lysozyme (added immediately prior to use).	2 $mg.ml^{-1}$
Solution II. (prepared freshly)	SDS	1 % w/v
	NaOH	0.2M
Solution III. (Stored at 4°C)	Glacial acetic acid	8.7 % w/v
	Potassium acetate	3.0 M
RF1 Buffer	RbC1	0.12%
	$MnC1_2.4H_20$	0.99 %.
	Potassium acetate	30 ml of a 1M stock (pH 7.5) per liter
	$CaCI_2.2H_20$	0.15 %.
	Glycerol	15%

The pH was adjusted to 5.8 with 0.2M acetic acid, made solution made up to one liter and filter sterilized by filtration through a 0.22μl filter membrane (Millipore). The RbCI and MnC12 were added after adjusting the pH.

RF2 Buffer		
	MOPS	20 ml of a 0.5 M stock (pH 6.8)/1.
	RbC1	0.12 %.
	$CaC1_2.2H_20$	1.1%.
	Glycerol	15 %

Dissolved in 800 ml of distilled water and pH adjusted to 6.8 with NaOH. Made up to one liter and filter sterilized by filtration through a 0.2212 Filter Membrane (Millipore). The RbC1 was added after adjusting the pH.

MLEE

Extraction Buffer (Haubold and Rainey, 1996)

0.1mg/ml	NADP
14mM	β-mercaptoethanol

Electrode Buffer (TG) Tris Glycine

30g	trizma base
144g	Glycine

Make up to 1 Litre.

Dilute 1:9 TG: ddH_2O for general use.

Examination and enumeration of polymorphic variations using MLEE of the enzymes GPI, G6PDH, ME, and 6PGDH were carried out using stains used by Haubold and Rainey (1996) for cellulose acetate gels

Glucose-6-phosphate isomerase (GPI)

25ml	TG buffer
5mg	NADP
10mg	Fructose 6-phosphate
5mg	MTT
1mg	PMS
10µl	G6PDH

Glucose-6-phosphate dehydrogenase (G6PDH)

25ml	TG buffer
5mg	NADP
20mg	D-Glucose-6-phosphate
5mg	MTT
1mg	PMS

Malate dehydrogenase NADP (ME)

Tris HCl, pH=8.0
NADP
Malic substrate
MgCl2
MTT
PMS**
Malic substrate
180ml dH2O
Tris HCl, pH=9.0
L-Malic acid Adjust pH=8.0

6-phosphogluconate dehydrogenase (6PGDH)

Tris HCl, pH=8.0
NADP
6-Phosphogluconic acid
MgCl2
MTT
PMS**

**Labile or photosensitive, add immediately before use

APPENDIX IV : BACTERIAL STRAINS USED IN THIS STUDY

Table 8-2 Bacterial strains used in this study.

Strain name	Genotype or description	Reference/source
***Escherichia coli* strains**		
S17-1	thi pro hsdR- hsdM+ recA RP4-2 Mu-Km::Tn7, StR TpR	(Simon, *et al.*, 1983)
S17-1 λpir	λpir thi pro hsdR- hsdM+ recA RP4-2 Mu-Km::Tn7, StR TpR	(Simon, *et al.*, 1983)
DH5α	supE44 ΔlacU169(ϕ80*lac*ZDM15) hsdR17 thi-1 recA1 endA1 gyrA96 relA1	(Hanahan, 1983)
DH5α λpir	supE44 ΔlacU169(ϕ80*lac*ZDM15) hsdR17 thi-1 recA1 endA1 gyrA96 relA1 λpir	(Hanahan, 1983)
***Pseudomonas* strains**		
P. tolaasii 2192T	B9, WL(R)+, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	NCPPB 2192T
P. tolaasii 2193	WL(R)+, sid+	NCPPB 2193
P. tolaasii 1116	B9, WL(R)+, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	NCPPB 1116
P. tolaasii 2325	B9, WL(R)+, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	NCPPB 2325
P. gingeri 3147T	B5, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	NCPPB 3147T
P. gingeri 3146	B5, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	NCPPB 3146
P. reactans 1311	B2, WL(T)+, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	NCPPB 1311
P. fluorescens B90	WL(T)-, WL(R)-, sid+	PAMS B90
P. putida B128	WL(T)-, WL(R)-	PAMS B128
P. aureofaciens PA147-2	WL(T)-, WL(R)-, sid+, Bfm+	(Godfrey, *et al.*, 2000)
P. fluorescens SBW25	Wild type, isolated from sugar beet plant, sid+	(Rainey, *et al.*, 1994)
Blotch Causing Organisms (BCOs) Pseudomonads isolated during Chapter 2		
NZ 032	B9, WL(R)+, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	This study
NZ 027	B9, WL(R)+, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	" "
NZ 006	B6, Bfm-, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 031	B4, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 009	B3, WL(T)+, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 014	B3, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	" "
NZ 024	B3, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 052	B9, WL(T)++, Bfm++, Prt++, Lip-, M9+, MJA+, Sid+	" "
NZ 062	B9, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 060	B4, WL(T)++, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 007	B4, WL(T)+, Bfm++, Prt+, Lip-, M9+, MJA+, Sid+	" "
NZ 081	B1, Bfm-, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 111	B9, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 065	B3, WL(T)+, Bfm-, Prt+, Lip-, M9+, MJA+, Sid+	" "
NZ 124	B8, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 066	B1, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 113	B3, Bfm+, Prt++, Lip+, M9+, MJA+, Sid+	" "
NZ 064	B1, Bfm-, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 102	B3, Bfm++, Prt-, Lip++, M9+, MJA+, Sid+	" "
NZ 097	B3, WL(T)+, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 101	B6, WL(T)+, Bfm++, Prt-, Lip++, M9+, MJA+, Sid+	" "
NZ 096	B2, WL(T)+, Bfm+, Prt+, Lip+++, M9+, MJA+, Sid+	" "
NZ 039	B3, Bfm++, Prt-, Lip++, M9+, MJA+, Sid+	" "
NZ 104	B3, Bfm++, Pr++, Lip+++, M9+, MJA+, Sid+	" "
NZ 017	B3, Bfm, Prt, Lip, M9+, MJA+, Sid+	" "
NZ 103	B9, Bfm++, Pr++, Lip++, M9+, MJA+, Sid+	" "
NZ 099	B1, Bfm+, Prt-, Lip-, M9+, MJA+, Sid+	" "

Strain name	Genotype or description	Reference/source
Blotch Causing Organisms (BCOs) Pseudomonads isolated during Chapter 2		
NZ 011	B6, Bfm++, Prt+, Lip+, M9+, MJA+, Sid+	" "
NZ 112	B3, Bfm++, Pr-, Lip-, M9+, MJA+, Sid+	" "
NZ 043	B5, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 059	B6, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
NZ 092	B5, Bfm++, Prt-, Lip++, M9+, MJA+, Sid+	" "
NZ 047	B5, Bfm++, Prt-, Lip-, M9+, MJA+, Sid+	" "
Tn5 generated NZ103 mutants		
P103A01	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, MJR; KmR	This study
P103B01	NZ103::mini-Tn5KmlacZ2, BfR, Lp-, Pr-, MJR; KmR	" "
P103D01	NZ103::mini-Tn5KmlacZ2, Bf-, MJR; KmR	" "
P103E01	NZ103::mini-Tn5KmlacZ2, Bf-, MJR; KmR	" "
P103G01	NZ103::mini-Tn5KmlacZ2, BfR, KmR	" "
P103A02	NZ103::mini-Tn5KmlacZ2, M9R, KmR	" "
P103G01	NZ103::mini-Tn5KmlacZ2, Bf-, KmR	" "
P103D02	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, MJR; KmR	" "
P103E02	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, MJR; KmR	" "
P103F02	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, MJR; KmR	" "
P103G02	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, M9R, MJR; KmR	" "
P103A03	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, KmR	" "
P103E03	NZ103::mini-Tn5KmlacZ2, Pr-, KmR	" "
P103F03	NZ103::mini-Tn5KmlacZ2, M9R, KmR	" "
P103G03	NZ103::mini-Tn5KmlacZ2, Bf-, Lp-, Pr-, M9R, MJR; KmR	" "
P103G03	NZ103::mini-Tn5KmlacZ2, MJR; KmR	" "
P103G04	NZ103::mini-Tn5KmlacZ2, Bf-, PrR, M9R, KmR	" "
P103A05	NZ103::mini-Tn5KmlacZ2, Bf-, Pr-, KmR	" "
P103B05	NZ103::mini-Tn5KmlacZ2, Bf++, KmR	" "
P103C05	NZ103::mini-Tn5KmlacZ2, Bf+, KmR	" "
P103D05	NZ103::mini-Tn5KmlacZ2, Bf+, KmR	" "
P103F05	NZ103::mini-Tn5KmlacZ2, MJ-, KmR	" "
P103*33	NZ103::mini-Tn5KmlacZ2, Pigment+, KmR	" "
P103A13	NZ103::mini-Tn5KmlacZ2, Lp-, KmR	" "
P103A14	NZ103::mini-Tn5KmlacZ2, Lp-, Pr-, KmR	" "
P103C10	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103C10	NZ103::mini-Tn5KmlacZ2, Pr-, KmR	" "
P103D11	NZ103::mini-Tn5KmlacZ2, PrR, KmR	" "
P103D13	NZ103::mini-Tn5KmlacZ2, Lp-, KmR	" "
P103D14	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103E10	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103E11	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103E15	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103F14	NZ103::mini-Tn5KmlacZ2, PrR, KmR	" "
P103G10	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103G11	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103G12	NZ103::mini-Tn5KmlacZ2, M9-, KmR	" "
P103G13	NZ103::mini-Tn5KmlacZ2, M9++, KmR	" "

IV(i) *P. TOLAASII* 2192 TRANSPOSON MUTANTS WITH ALTERED BIOFILM PHENOTYPES

Table 8-3 *P. tolaasii* NCPPB2192 mini-Tn*5*km*lacZ2* mutants exhibiting altered abiotic biofilm formation obtained during this study and cryogenically stored. Biofilm refers to abiotic biofilm on PVC, 12 hrs, 28°C in L10 media.

-80oC label	Plate Isolate	Biofilm
PTB03	T23D2	-
PTB04	T23D4	-
PTC02	T224c11	-
PTD04	T21C3	-
PTE02	T20C11	-
PTE03	T21C12	-
PTE04	T20E12	-
PTF01	T19C1	-
PTF02	T19D2	-
PTF03	T19F3	-
PTG02	T18C11	-
PTG06	T18B3	-
PTH03	T18E5	-
PTA14	T16C11	-
PTA15	T16B10	-
PTB12	T15B4	-
PTB13	T15D2	-
PTC12	T14D9	-
PTC13	T14E9	-
PTD12	T13C1	-
PTD13	T13D2	-
PTD14	T12B10	-
PTE14	T12E10	-
PTF13	T11B1	-
PTF14	T11C5	-
PTG12	T11G2	-
PTG13	T11E4	-
PTH11	T10G12	-
PTH12	T08F12	-
PTH13	T08D9	-
PTB21	T09B4	-
PTB25	T09F3	-
PTC22	T03B4	+
PTC23	T03D3	-
PTD24	T03A4	-
PTE22	T03F4	++
PTE23	T03G2	++

APPENDIX V : PLASMID VECTORS USED DURING THIS STUDY

V(i) COMMERCIALLY AVAILABLE VECTORS

Designation	Description	Reference
pBluescript	ColE1 ori *lac*Za/SK polylinker (polylinker in opposite orientation for KS); Ap^R	(Short *et al.* 1988)
pBR322	ColE1 ori ApR TcR (4361 bp), Tc^R	(Bolivar, *et al.*, 1977)
pUTZ2	pUT containing mini-Tn*5*km*lac*Z2	(de Lorenzo, *et al.*, 1990)
pGEM-T	Cloning vector for PCR products, Ap^R	Promega

APPENDIX VI: OLIGONUCLEOTIDE AND PCR CONDITIONS

VI(i) OLIGONUCLEOTIDE PRIMERS USED DURING THIS STUDY

Table 8-4 Oligonucleotide primer sequences used during this study.

Name		Oligonucleotide sequence	Source / Reference
16S rRNA			
PA	5'-	AGAGTTTGATGMTGGCTCAG	Lane *et al.*, 1991
519R	5'-	GWATTACCGCGGCKGCTG	" "
PHI	5'-	AAGGAGGTGWTCCARCC	" "
Universal R	5'-	GYTACCTTGTTACGACTT	" "
U16a	5'-	AGAGTTTGATCCTGGCTC	" "
U16b	5'-	TACGGYTACCTTGTTACGACTT	" "
16F1103	5'-	TGTTGGGTTAAGTCCCGCAAC	" "
16F945	5'-	GGGCCCGCACAAGCGGTGG	" "
16R518	5'-	CGTATTACCGCGGCTGCTGG	" "
16F357	5'-	ACTCCTACGGGAGGCAGCAG	" "
16R1087	5'-	CTCGTTGCGGGACTTAACCC	" "
pvd1 and pvd2			
pvd1	5'-	GTCAGCGTCCAGCCCTTG	This study
pvd2	5'-	GTGCTCAGCCGTGCCTAC	" "
			" "
REP-PCR			
REP2-1	5'-	ICGICTTATCIGGCCTAC	de Bruin, 1992
REP1R-1	5'-	IIIICGICGICATCIGGC	" "
Primers for commercial Vectors			
T3	5'-	ATTAACCCTCACTAAAG	pBluescript II SK- (Promega)
T7	5'-	AATACGACTCACTATAG	" "
SP6	5'	GATTTAGGTGACACTATAG	" "
***LacZ* – *Tn5* PCR investigations**			
Tn5-O		ACTTGTGTATAAGAGTCAG	This study
Lac_no.18		CCGTCGTTTTACAACGTCGT	" "
pBR-Pst-For		GCTTACCATCTGGCCCCR	" "
pBR-Pst-Rev		TGACAACGATCGGAGGACC	" "

VI(ii) PCR REACTION CONDITIONS

Clean RNA free DNA was used as the substrate in PCR reactions. Unless stated otherwise, 25μl PCR reactions were set up as follows in 0.2ml PCR tubes:

VI(ii)a PCR programs

Program:	Touchdown 1	Touchdown 2	Touchdown 3
Step 1.	94°C 5 min	94°C 5 min	94°C 5 min
Step 2	94°C 30 sec	94°C 30 sec	94°C 30 sec
Step 3	60°C 30 sec	60°C 30 sec	60°C 30 sec
Step 4	72°C 1 min	72°C 2 min	72°C 3 min
Step 5	2x Repeat steps 2-4	2x Repeat steps 2-4	2x Repeat steps 2-4
Step 6	94°C 30 sec	94°C 30 sec	94°C 30 sec
Step 7	57°C 30 sec	57°C 30 sec	57°C 30 sec
Step 8	72°C 1 min	72°C 2 min	72°C 3 min
Step 9	2x Repeat steps 6-8	2x Repeat steps 6-8	2x Repeat steps 6-8
Step 10	94°C 30 sec	94°C 30 sec	94°C 30 sec
Step 11	55°C 30 sec	55°C 30 sec	55°C 30 sec
Step 12	72°C 1 min	72°C 2 min	72°C 3 min
Step 13	2x Repeat steps 10-12	2x Repeat steps 10-12	2x Repeat steps 10-12
Step 14	94°C 30 sec	94°C 30 sec	94°C 30 sec
Step 15	50°C 30 sec	50°C 30 sec	50°C 30 sec
Step 16	72°C 1 min	72°C 2 min	72°C 3 min
Step 17	30x Repeat steps 14-16	30x Repeat steps 14-16	30x Repeat steps 14-16
Step 18	72°C 5 min	72°C 5 min	72°C 5 min
Step 19	25°C 7 min	25°C 7 min	25°C 7 min
Step 20	10°C ∞	10°C ∞	10°C ∞

Program:	16S	Rep PCR	Whole Cell Lysis
Step 1.	94°C 5 min	94°C 6 min	99.9°C 10 min
Step 2	94°C 30 sec	94°C 1 min	25°C 10 min
Step 3	55°C 30 sec	40°C 1 min	10°C ∞
Step 4	72°C 1 min	65°C 8 min	
Step 5	30x Repeat steps 2-4	30x Repeat steps 2-4	
Step 6	72°C 5 min	65°C 16 min	
Step 7	25°C 1 min	10°C ∞	
Step 8	10°C ∞		

APPENDIX VII

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